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INTERNATIONAL ENVIRONMENTAL AMR FORUM – DRAFT REPORT

Prepared by:

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Human and Animal Contamination

I.   BACKGROUND STATEMENT

Bacteria that cause infections in humans and animals are becoming increasingly resistant to antibiotics. In addition to causing infection, these bacteria can asymptomatically colonize a host, often in the gastrointestinal tract. As a result, disposal of waste from a colonized human or animal can become a source of resistant bacteria in the environment. These sources of contamination will become more important as the problem of resistance increases. Once resistant bacteria are in the environment, they have the potential to spread to new populations; colonizing new hosts (humans or animals) and causing new infections. Of special concern are resistance in bacteria known to cause human infections and bacteria carrying easily mobilized resistance determinants (e.g., resistance genes on plasmids) that confer resistance to medically important antibiotics.

Human and animal waste can also be a source of medically important antibiotics in the environment. If these antibiotics are present and active, they may apply selective pressure on the microbial population resulting in an amplification of any resistant bacteria that may be present.

Understanding the risk to human health from environmental contamination of resistant bacteria requires further research and data collection in the environment. This work should be performed using methods and sampling strategies that determine which type of resistance is present, the quantity of resistant bacteria, the source of contamination (i.e., attribution), and the extent to which the resistance has spread (or disseminated). Research methods and data collection should also be suitable to measuring the impact of interventions used to prevent or remove environmental contamination.

Responding to environmental contamination of resistant bacteria could include prevention strategies (e.g., treating sewage before it is released) and removal strategies (e.g., waste water treatment processes). It is important to understand the effectiveness of existing practices for waste management and water processing, as well as investigating novel methods and strategies.

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II.   SCIENTIFIC ISSUES

A. Contamination of the Environment

To what extent are human waste or animal waste contaminating the environment with AR pathogens?

i.   Hospitals

Locations of greater antibiotic use, especially antibiotics of clinical importance, and/or releases of potential co-selecting agents deserve particular attention, such as hospitals, clinics, and other healthcare facilities.

There are four issues that should be considered relative to AR pathogens/bacteria within and released from healthcare facilities:

1.   Antibiotic use and the release AR bacteria and antibiotics in urine and fecal matter that enters the facility wastewater collection system.

2.   Presence of AR bacteria within the facility plumbing system, such as sinks, taps and other sources of water.

3.   Clinical waste disposal where appropriate solid waste processing facilities are not available; and

4.   In-house sanitation conditions, which can be particularly problematic in facilities in less developed countries or more rudimentary environments.

All these factors impact AR pathogen/bacteria releases in wastewater from a healthcare facility, but the relative influence of each factor depends on the nature, size, management, and location of the facility. For example, wide differences exist in the handling and disposal of hospital wastewaters. And, depending upon the country, healthcare facilities may or may not be required to have their own wastewater treatment plants. European Directive 91/271/EEC states that hospital wastewater can be discharged to sewers without further treatment, whereas hospital wastewater treatment is mandated in India.

It should be noted that the majority of current regulations were developed before the role of the environment to the global AR problem was known. Therefore, an assessment on whether specific wastewater treatment should be considered for healthcare facilities as part of a wider strategy to mitigate against AR pathogen/bacteria in the environment is found later in this report.

Characteristics of healthcare facility wastewaters

There is evidence that the concentrations of many bacteria are similar in urban and hospital wastewater, but the proportion of resistant bacteria are higher in hospital effluent. This has been demonstrated for vancomycin resistant enterococci (VRE), which were significantly more prevalent in hospital compared to community effluent (Hocquet, 2016; Varela, 2013; Varela, 2015). In Bangladesh, the prevalence of NDM-1-producing bacteria and bla_{NDM-1} genes in wastewater samples adjacent to hospitals was significantly higher than in community wastewater samples from the same city (71% vs 12.1%; Islam, 2017).

Antibiotic residue concentrations in hospital effluent have, in some cases, been found to correspond with the most common antibiotics used in hospitals, for example, ciprofloxacin use and ciprofloxacin concentrations in hospital effluent in India were correlated (Diwan, 2010) but the effect of these antibiotics on *E. coli* isolates in water was not clear. Furthermore, there is growing evidence that, although the abundance of AR pathogens/bacteria in hospital wastes has been reported to be not more than ten times higher than community wastes, AR pathogens/bacteria from hospitals tend to carry more AR genes per cell (Quintela-Baluja, submitted), especially in gram(-) *Enterobacteriaceae* with potentially promiscuous plasmids.

Stecher (2014) showed that gut inflammation can accelerate horizontal gene transfer (HGT) and an elevated potential for HGT may be a common trait in intestinal pathogens, which may be more prevalent in hospital wastewaters. Although these data are for a model system, such a trait may further allow organisms to more readily exchange AR genes to both commensal and environmental bacteria once they enter the community wastewater stream (Chamosa, 2017). This potentially explains why multidrug resistance (MDR) organisms sometimes pass through sewer systems and wastewater treatment plants, and are detected in downstream receiving waters. Therefore, the abundance of AR pathogens/bacteria may not always be elevated in healthcare facility wastes compared to community wastes, but the bacteria themselves appear to have levels of resistance and also be more prone to HGT.

The above information is based on limited studies, and as such represents another major knowledge gap that is central to the debate about whether healthcare facility wastes should be treated at source or not. There is no absolute proof that greater MDR AR pathogens in hospital wastes pose a greater risk than comparable organisms from the community. However, evidence hints that such organisms from healthcare facilities are prone to greater HGT and have higher minimum inhibitory concentrations (MICs). Therefore, more work is needed to determine the potential risk to health.

Drivers of AR Pathogens/Bacteria within Healthcare Facilities

Selective drivers of AR in pathogens or commensal bacteria in hospitals include antibiotic use; the relative health status of treated patient; and opportunities for transmission and exchange of AR bacteria, genes, and mobile genetic elements among patients, caregivers, and healthcare professionals. Common multi-drug resistant bacteria recovered from hospital wastewater include ESBL-producing *E coli*, vancomycin-resistant enterococci and *Pseudomonas aeruginosa* (Hocquet, 2016).

Although healthcare facilities are locations of greater antibiotic use (i.e., 20-30% of inpatients in acute care European hospitals receive antibiotics) (Ansari, 2009), antibiotics themselves are not the only driver of AR bacteria selection and transmission in facilities. Patients may carry AR bacteria and genes before entering hospital, acquire them during their inpatient stay, and/or have AR bacteria selected without their gut or urine as a direct result of antibiotic therapy (Salm, 2016; Weingarten, 2018). As such, AR pathogens are often only detected when therapeutic treatment fails or clinical symptoms worsen. Hence, AR bacteria and genes might be present in the gut of a patient and not detected, and released in wastewater independent of local antibiotic use (Finlay, 2013).

Similarly, AR genes or bacteria detected in a healthcare facility do not necessarily originate from within the facility, particularly in locations where background human, animal, or environmental prevalence of AR bacteria is comparatively high (Graham, 2014). Therefore, ascribing the root cause of detected AR bacteria in a given wastewater is often hard to achieve; this is a major knowledge gap in understanding AR in wastewaters from healthcare facilities.

In summary, AR pathogens/bacteria can either be in patient wastes (acquired before or within the facility) or within the healthcare facility local environment. Regardless of source, antibiotic use further selects for AR bacteria and genes, which are readily detected in facility wastewaters and often correlate (Varela, 2014). However, antibiotic use and levels within a facility do not always correlate with AR bacteria or gene levels in a facility wastewater. This is partly because antibiotics and AR bacteria/genes have different attenuation mechanisms after release to the environment. Rates of antibiotic degradation are highly variable and depend on the specific antibiotic and receiving environment with half-lives ranging from minutes to tens of days (Homem, 2011). The relationship between antibiotics and AR bacteria/genes also depends on global locations with different environmental temperatures and different AR colonisation rates within the wider community.

Co-mingling of healthcare facility wastewaters and community wastewaters

The point of co-mingling between healthcare facility wastes and wastes from the wider community appears important to the type and nature of AR bacteria that move further downstream in sewer systems ultimately to WWTPs (Quintela-Baluja, 2018). Bacteria are known to display accelerated HGT when under stress; therefore, changes in their local habitat influence the rates at which they exchange genes and evolve, including sharing AR genes. HGT at the co-mingling point in the sewers is impacted by differences in temperature, the presence of co-selective metals and biocides, and basic differences among bacteria from healthcare versus community versus environmental sources.

However, there is debate about the relative importance and differences between hospital and community waste streams (Wang, 2018). Early findings suggest that healthcare-borne bacteria have a greater potential for HGT and may have selective advantages that enhance their survival in wastewater treatment, although more data are needed to confirm this observation. A key knowledge gap is whether bacterial isolates from hospital wastewaters are fundamentally different to bacteria in community wastewaters because there is currently no method of distinguishing between isolates from different sources. This methodological gap makes it difficult to determine the specific risk of healthcare facility wastes.

ii.　　Human sewage

To what extent is human waste contaminating the environment with AMR?

Human sewage contains pathogenic and commensal enteric bacteria carrying genes for resistance to antibiotics. Many potentially disease-causing bacteria, including *Escherichia coli, Klebsiella pneumoniae* and *Acinetobacter baumannii,* colonize in the GI tract of animals and humans and, when resistant, contribute to AR bacteria in human sewage (Sobsey, 2014). For example, E. coli is of concern for community-associated antibiotic resistance due to its natural occurrence in humans, animals and the environment, as well its association with highly transmissible resistant mechanisms such as extended-spectrum beta lactamase (ESBL) production via *bla*NDM-1 genes conferring resistance to carbapenems (Sobsey, 2014). Globally, an estimated 14% of healthy humans are colonised by ESBL-producing Enterobacteriaceae (ESBL-PE) with prevalence rates as high as 22% in Southeast Asia and Africa (Karanika, 2016). These and other bacteria contribute to the environmental resistome through their release into sewage, wastewater and subsequently onto land or surface waters.

Traditional wastewater treatment plants (WWTPs) are not designed for the removal of antibiotic resistance genes from human waste and can actually provide favourable conditions for the amplification of resistance genes through the interaction of antibiotic residues and enteric bacteria via horizontal gene transfer on mobile genetic elements (Pruden, 2013). Antibiotic resistance genes can persist even in advanced WWTPs and remain at detectable levels in surface waters receiving the discharge (Singer, 2016). The subsequent introduction of resistant bacteria and genes into recreational and coastal bathing water increases exposures to humans and wildlife (e.g. shellfish or birds) (LaPara, 2011).

Untreated human waste, therefore, poses a significant risk of resistance transmission in the environment. The weak sanitation infrastructure in many urban centres around the world means that only a proportion of human sewage is appropriately treated (e.g. 56% in Delhi, India; 55% in Kumasi city, Ghana). For example, in Dhaka, Bangladesh, only 1% of human excreta is treated effectively whilst 70% is discharged directly into the environment (Peal, 2015).

In high-income countries with well-developed sewage infrastructure, the discharge of antibiotics to the environment is reduced. Within treatment plants, however, microbial communities can be exposed to higher concentrations of antibiotics. At least 56 antibiotics belonging to six different classes have been detected at nanogram-per-liter to microgram-per-liter levels in the influent and effluent of WWTPs in East Asia, North America, Europe, and Australia, corresponding closely with the most commonly prescribed antibiotics for human use (Zhang, 2011). Such levels are not considered to present significant human health risk since they fall well below the therapeutic dose (WHO, 2012). However, in many LMICs the concentrations of antibiotic residues have not been assessed and may be higher. Recent studies have demonstrated the surprising environmental prevalence of human waste contamination in rural areas from sources like septic systems (Verhougstraete, 2015) and in urban areas from sources like stormwater outfalls (Sauer, 2011).

There are emerging concerns around the use of treated sewage sludge (biosolids), largely composed of human waste, on agricultural land. Treated sewage sludge in Europe has been found to contain trace levels of antibiotics, resistance enzymes such as beta-lactamases, as well as ESBL resistance genes, demonstrating that standard treatment is not sufficient to remove these (Wellington, 2013). There is currently very limited understanding of the downstream consequences of these trace chemical and biological contaminants, as research in this field is sparse and needs further investigation of the potential for transfer of resistance to soil bacteria and onward transmission to human-associated bacteria.

What strategies should be employed for tracking AR pathogen or antibiotic contamination from each source?

Human wastes, as well as environmental reservoirs of antimicrobial resistance could be significant sources for the emergence and spread of resistance, and they need to be better understood. Priority antimicrobials, resistance genes, and resistant microbes should be identified for tracking strategies. E. coli is a common sentinel organism for monitoring the prevalence of resistance in humans, livestock, and the environment, but the priority organisms or genes may vary depending on the focus of resistance tracking (WHO, 2017). Once these are identified, surveillance strategies should consider aspects like the temporal and spatial nature of the risk to human and animal health (Vikesland, 2017).

There are a variety of research tools that can form a toolbox for the detection and source tracking of antimicrobial chemicals, antimicrobial resistant microorganisms, and antimicrobial resistance genes from sources such as human wastes. Some of these tools can be applied directly to detection and source tracking of specific antimicrobial chemicals, antimicrobial resistant microorganisms, or antimicrobial resistance gene targets. In particular, advances in whole genome sequencing and metagenomics technologies, both discussed in more detail below, offer growing opportunities to better understand the evolution and transmission of antimicrobial resistance, the epidemiology of antimicrobial resistant pathogens, and the significance of sources of fecal pollution such as human wastes for the spread of antimicrobial resistance (Martinez, 2017). Other tools like microbial source tracking (Harwood, 2014) can be applied indirectly to detect human fecal waste in the environment as part of multiple lines of evidence to infer the source of antimicrobial chemicals, resistant microorganisms, and resistance genes.

Where there are risks to human health, animal health or the environment from antimicrobial chemicals, resistant microorganisms, or resistance genes, environmental surveillance strategies should consider the need to better characterize aspects like the temporal and spatial nature of risks. An environmental monitoring strategy based on use of standardized operational methods should be considered when assessing risk, starting at sentinel or other key sites to enable assessment of longer-term trends in antimicrobial resistance concerns associated with human wastes and the environment. Once the risks to health are established, data from research, surveillance and monitoring strategies should ultimately be used to identify and evaluate interventions and risk management options for reducing antimicrobial resistance concerns associated with human wastes.

iii.   Animal farms

Are wastes generated or used in agriculture a source of AMR?

Antibiotic resistance genes conferring reduced susceptibility to multiple classes of antibiotics are abundant in animal, poultry, and fish manures, as well as biosolids that are used to fertilize agricultural land (Zhu, 2013; Wang, 2017; Muziasari, 2017; Brooks, 2014; Cook, 2014). These occur in the absence of selection but are no doubt selected or co-selected for by antibiotics and other antibacterial agents (i.e. metals, biocides and detergents) that are commonly applied in food animal production systems (Chantziaras, 2014; Hoelzer, 2017; Pal, 2015; Pal, 2017; Hu, 2016; Johnson, 2016). Culture-based and culture-independent studies indicate that antibiotic resistant genes (ARGs) can be found in bacteria that are pathogenic for humans, and in non-pathogenic "reservoir" bacteria. Data from the U.S. National Antimicrobial Resistance Monitoring System (NARMS), a culture-based nationwide surveillance effort focused on AR in humans, fresh retail meat products, and food animals, show that resistance in bacteria causing foodborne illness has declined or held steady for over a decade (FDA, 2015). However, NARMS does not track AR in reservoir bacteria.

Antibiotic resistance genes (ARGs) are often carried on mobile genetic elements such as plasmids that are amenable to horizontal gene transfer. For example, IncA/C plasmids carrying the resistance gene *bla*CMY-2 are widely distributed in Salmonella and Enterobacteriaceae in North American cattle (Mollenkopf, 2017). They may also be transferred via bacteriophage (Mirzaei, 2017). Once transferred into a bacterium, the genes are spread vertically via bacterial replication. While all bacterial groups can carry antibiotic resistance genes, some groups (Clostridia) have been identified as potentially more relevant in terms of environmental transfer and human/animal health (Durso, 2012; Scott, 2018; Leclercq, 2016).

When antibiotics are administered to food animals, they are generally excreted intact, although they have highly variable persistence in the environment. Antibiotics have been shown to increase the incidence of horizontal gene transfer of ARGs in animal intestines (Bearson, 2014; Chambers, 2015). Those ARGs from livestock and the mobile genetic elements on which they are carried disseminate and persist on land after manure application (Pornsukarom, 2017). There are concerns that excreted antibiotics and their bioactive breakdown products provide a selective force for enrichment of resistance in the soil, and that residue-laden land-applied manures alter the structure of soil microbial populations in ways different from antibiotic-free manures (Jechalke, 2014; Liu, 2016).

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Are environments exposed to agricultural wastes contaminated with AMR?

The methods of processing agricultural manures vary depending on many factors, including the specific commodity, the size of the operation, the soil type, and proximity to surface and ground water (Durso, 2017). In confined production systems, manures may be treated through aerobic (eg. composting) or anaerobic digestion prior to application. The distribution and abundance of antibiotic resistant bacteria and resistance genes can be altered profoundly by these treatments; but their efficacy at reducing environmental exposure is not yet firmly established (Wolters, 2015; Xie, 2016). Soils fertilized with animal manures or biosolids are enriched in antibiotic resistant bacteria (Pornsukarom, 2016) and resistance genes (Pornsukarom, 2017) compared to soils that do not receive animal manures, regardless of whether or not the animals have received antibiotics (Udikovic-Kolic, 2014). Once in soils, either because they are part of the baseline resistome or added via manure application, the genes may persist even in the absence of direct drug selection pressure (Kyselkova, 2015). Many studies show that manure amendments lead to increase AMR in the soil, (Cook, 2014; Fahrenfeld, 2014; Marti, 2014; Williams-Nguyen, 2016; Muurinen, 2017), with the potential to contaminate crops. (Marti, 2013, Rahube, 2014).

Commercial manure application rates that are calibrated to crop agronomic needs will entrain perhaps 108 to 1013 copies of each ARG per hectare (Tien, 2017). Following application, antibiotic resistance genes can persist for months, but their viability and the impact of key rate controlling factors such as climate and soil characteristics remains to be systematically defined (Fahrenfeld, 2014; Marti, 2014). In a survey of 40 publications measuring AR genes in agricultural wastewater, when normalized to the 16S gene, the majority of values fell within the 10-2 to 10-3 range, representing one AR gene for every 100 to 1000 16S genes in the samples (Durso, 2017).

The detection of carbapenem-resistant bacteria in feces or the production environment of cattle, swine and poultry is particularly worrisome; widespread human exposure via the environment or food supply could potentially compromise this critically important class of antibiotics. (Poirel, 2012; Al Bayssari, 2015; Webb, 2016). Surface and groundwater resources in proximity to livestock production and areas of manure application are subject to contamination with antibiotic resistance genes. (Chen, 2011; Coleman, 2013). Marine sediments in areas where aquaculture is practiced are enriched in antibiotic resistance genes and mobile genetic elements. (Gao, 2010; Muziasari, 2016). The additional burden of ARGs needs to be placed within the context of the environmental resistome that is normal (Allen, 2010; Cytryn, 2013; Durso, 2014; Rothrock, 2016).

What strategies should be employed for tracking AMR or antibiotic contamination from agriculture?

There are currently no environmental AMR surveillance initiatives undertaken by any jurisdiction. Existing AMR surveillance models such as NARMS that focus on specific target bacteria with clinically-important resistance phenotypes may be inadequate for environmental AMR surveillance. Research initiatives tracking AMR consist of culture-dependent and independent detection and quantification of AMR bacteria and gene targets associated with resistance or mobility. It has been suggested that int1 is a useful indicator of anthropogenic AMR contamination. (Gillings, 2015). Faecal Clostridium spp. and soil Acinetobacter and Pseudomonas spp. may be important for the spread of resistance genes. (Leclercq, 2016). Field experiments have evaluated the movement of AMR genes and bacteria from ground receiving manure to crops (Wang, 2017; Tien, 2017; Marti, 2013; Lau, 2017), as well as animal products that might be impacted, such as from aquaculture or game animals.

The environment is subject to fecal contamination from agriculture, humans, and wildlife. Ascribing the relative importance of point or (in particular) non-point contamination sources is a challenge, particularly in light of natural and baseline levels of AR in the environment (Durso, 2014; Rothrock, 2015). For aquatic environments this can be done by georeferencing with land use practice and intensity, co-localizing antibiotic resistance with fecal source tracking markers (ie. fecal-source specific bacteroidales, mitochondria), molecular epidemiology, and modelling source intensity/transport pathways/persistence. (Teaf, 2018).

Quantitative modelling could clearly play a role in informing and developing policy, both in terms of general agricultural practise, and in response to specific outbreaks. Quantitative and non-quantitative risk models are already used for food safety in relation to microbial pathogens (e.g. Romero-Barrios, 2013). Environmental AR present unique challenges in quantifying direct vs indirect risk, and navigating the complexities of the relationship between AR bacteria and AR genes. (Williams-Nguyen, 2016). More generally, mathematical models can be used for sensitivity analyses in order to identify those factors through which spread of resistant bacteria and/or resistance genes can be best controlled (Baker, 2016).

However, there are considerable challenges in developing useful, realistic, dynamical models for antimicrobial resistance (Zhu, 2017). First, antimicrobial resistance is not a single phenomenon, but encompasses a wide range of organisms, genes, mobile genetic elements and selective agents; models will need to encompass this complexity. Second, AMR occurs on a wide range of scales, from gene

transfer events in single cells, through to major changes in whole ecosystems; models will need to operate on multiple scales. Third, to calibrate models against real data, there will need to be agreed standards for data capture and sharing, and the development of global databases for storing such data. The NARMS efforts are an example of a long-term AR surveillance program with harmonized data collection and reporting. Given the complexity of environmental AR, a valuable alternative modelling approach may be to use Bayesian network based methods (Beaudequin, 2015). Bayesian networks describe dependences between different outcomes using probabilities, so can model complex interacting systems in order to quantify risks of adverse events occurring. They naturally allow for uncertainty and gaps in knowledge, and can also be used to identify critical control points for interventions.

Overall, an important overriding question is what methodologies to monitor and track AMR in the environment will be most informative with respect to informing human and animal health risk, and benchmarking potential regulatory standards.

B. Impact on Human Health

Once environmental waters are contaminated, what evidence exists that this results in the spread of AMR resulting in an increased threat to human health?

Data do support an increased risk to human health or attributable mortality of AMR pathogens over susceptible pathogens (Roberts, 2009; de Kraker, 2011a; de Kraker, 2011b). However, more research is needed to provide estimates of health impact from exposure to AMR in environmental sources. Antibiotic resistant bacteria have been detected in environmental waters at exposure-related sites in different studies (Huijbers, 2015). For example, probable exposure was shown for swimmers to ESBL-producing Enterobacteriaceae (Schijven, 2015). There are six main routes of transmission of AMR from environmental waters to humans: I) recreational water, II) water used for drinking and washing, III) consumable fish and bivalves, IV) produce contaminated with either treated or non-treated surface water* (Ashbolt, 2013; Huijbers, 2015), V) urban waters, and VI) wastewater.

Recreational exposure

In 2003, the global burden of illness both from wastewater release into coastal environments and acquired by swimming or shellfish consumption was estimated at 120 million cases of gastrointestinal (GI) disease and 50 million cases of respiratory disease (Shuval, 2003). A recent systematic review on health outcomes associated with recreational coastal bathing water exposure in

Organisation for Economic Co-operation and Development (OECD) countries concluded that there is an increased risk of experiencing symptoms of any illness [odd ratio (OR) = 1.86, 95% confidence interval (CI): 1.31 to 2.64, P = 0.001] and ear ailments (OR = 2.05, 95% CI: 1.49 to 2.82, P < 0.001) in bathers compared with non-bathers. There is also an increased risk of experiencing GI ailments (OR = 1.29, 95% CI: 1.12 to 1.49, P < 0.001) [21]. As the burden of ARB and ARGs increase in wastewater, an increasing proportion of these infections will be caused by ARB.

Recreational waters (and associated beach sands) are increasingly recognized as a reservoir of ARB and ARGs, and important in the development of antibiotic resistance in pathogenic bacteria. Below are studies evaluating antibiotic resistance in recreational waters, which highlight several resistance genes and organism types found in both fresh and marine waters. However, comparisons between studies are difficult to make as the geography, ARGs selected for evaluation, sources of fecal impacts, and methods of determining resistance are highly variable from study to study (Alm, 2014).

Prospective cohort epidemiological studies on three California beaches correlated the detection of a variety of indicators, ARB, and pathogens with incidence of GI illness (Griffith, 2016). Methicillin-resistant *Staphylococcus aureus* (MRSA) was highly associated with GI illness, showing a stronger correlation than EPA's current culture EPA Method 1600 at the beach where it was measured, which was impacted by human sewage from faulty infrastructure (Griffith, 2016). This work highlights that recreators could in some cases be exposed to MRSA in waters impacted by human sewage. Separately, the prevalence of *S. aureus* and MRSA in ten freshwater beaches in Northeast Ohio was evaluated (Thapaliya, 2017). The overall prevalence of *S. aureus* in sand and water samples was 22.8% (64/280). The prevalence of MRSA was 8.2% (23/280). The highest prevalence was observed in summer (45.8%; 55/120) compared to fall (4.2%; 5/120) and spring (10.0%; 4/40). The results of this study indicate that beach sand and freshwater of Northeast Ohio were contaminated with *S. aureus*, including MRSA. The high prevalence of *S. aureus* in summer months and presence of human-associated strains may indicate the possibility of role of human activity in *S. aureus* contamination of beach water and sand.

A case-control study evaluating the risk factors for community acquired ESBL-positive urinary tract infections (UTI) identified recreational freshwater swimming within the past year as one of several independent risk factors (OR = 2.1; 95% CI: 1.0–4.0) (Soraas, 2013). Thus the study suggests swimming may be a risk factor for intestinal colonization with ESBL-positive *E. coli* and any subsequent UTI may be caused by a newly acquired ESBL-producing strain from the water. However, authors noted this

particular environmental link needs to be substantiated with more evidence. MDR E. coli were found omnipresent in surface waters used for recreation e.g. ESBL-producing *E. coli*. From these data, using a risk assessment framework, it could be concluded that exposure to ESBL-producing *E. coli* by swimming is likely and ingests at least one ESBL-producing *E. coli* when recreational waters are located downstream of WWTPs or livestock farms. More research is warranted for the evaluation of public health effects, such as colonization, infection, or horizontal gene transfer, upon exposure (Schijven, 2015). Attempts have also been made to derive population-level exposure estimates to third generation cephalosporins (3GCs) resistant *E. coli* (3GCREC) during marine recreational water use in England and Wales (Leonard, 2015). Authors estimated the prevalence of the 3GCRECs in coastal recreational waters, combined the data with the *E. coli* density from coastal beaches, and applied the information to ingestion volume estimates for various recreational activities. Together, the data resulted in the mean number of 3GCREC ingested during different water sports. Despite a low prevalence of 3GCREC (0.12%), the authors noted there is a human exposure risk for water users, which can vary by water sport activity.

Additional work has focused on all ARGs carried by *E. coli* using a targeted metagenomic approach, where pooled *E. coli* isolates from cultures derived from routine bathing water quality testing by the UK Environment Agency were sequenced and data analysed for presence and relative abundance of ARGs (Leonard, 2018a). Using these data, it was estimated that in 2016 in England every bathing water exposure event involved ingestion of at least one ARG associated with *E. coli* and that 2.5 million exposure events occurred involving ingestion of at least 100 *E. coli* borne ARGs. Using such approaches, an exposure risk assessment can be conducted, but health impact assessment is not possible due to a lack of dose-response data in terms of colonization and/or infection with ARB. For known pathogens where dose-response data is available, this may be possible but for ingestion of ARG-bearing opportunistic pathogens or other non-pathogens, the number of bacteria ingested related to the probability of colonization, 13obilization of ARGs to the gut microbiome, or probability of infection are unknown.

Finally, efforts have been made to assess risk of gut-colonization in highly exposed coastal bathing water users, i.e. surfers who ingest large volumes of water. A cross-sectional epidemiological study was conducted comparing regular surfers and non-surfers to evaluate the association between water exposure and gut colonization by 3GCEC. Results indicated that 6.3% of surfers were colonized by 3GCEC, compared to 1.5% of non-surfers (risk ratio = 4.09; CI 1.02-16.4) (Leonard, 2018b). Numerous studies demonstrate that colonization with ARB places humans at increased risk of infection; evidence of

this relationship is most abundant in healthcare, where attack rates of infection are greater and the time span between colonization and infection may be quite narrow (Tacconelli, 2009). Given this relationship, there are important aspects regarding the risk for colonization of the human GI tract that bear mention. The first is the natural colonization resistance afforded by the mature human microbiome and how this is disrupted by antibiotics and other environmental exposures, leaving individuals more susceptible to colonization by ARB. Thus, hospitalized and recently hospitalized or debilitated patients with chronic illness are more susceptible to colonization. In addition, infants and young children, due to their immature microbiome, are an important sentinel population in the community for environmental risks from AMR transmission.

In addition to the loss of colonization protection from an intact microbiome, ongoing high-level exposure to environmental ARB may result in transient or apparently persistent colonization—this is likely the case with the healthy surfers and individuals in the community with ongoing exposure. Evidence that removal of the ongoing exposures will result in slow clearance can be seen in healthy travelers who return colonized from settings where there were, presumably, intense environmental (i.e. water, food) exposure. This colonization typically 'clears' over several months and may be associated with not only the exposure to the ARB/ARGs but a likely microbiome-disruptive event such as abrupt dietary and travel-related stress changes.

Potable water

Coleman et al. demonstrated that having AMR *E. coli* in the home potable water supply was independently associated with carriage (Coleman, 2012). Under poor water, sanitation, and hygiene conditions, AMR can be present in water intended for human consumption (e.g. Walsh, 2011). In regions under higher hygienic standards, antibiotic resistant bacteria, genes and antibiotics have been detected in source waters for drinking water production. With risk assessment and risk management frameworks for safe drinking water production in place such as the WHO Water Safety Plans, risks from drinking water consumption should be controlled, but this should be evaluated [ref].

Fish and bivalve consumption

Antibiotic resistant fish pathogens have emerged in the aquaculture environment with evidence of transfer of resistance determinants to bacteria carried by terrestrial mammals, including humans (Cabello, 2006).   Determinants for resistance to tetracyclines, fluoroquinolones and beta-lactams are examples with exchange observed between *Aeromonas* and *E. coli* (Cabello, 2013).

Wastewater

Whilst there are major gaps in the literature with respect to precise measurement of burden of illness of AMR infections acquired from the environment (Varela, 2013), it is clear that antibiotic resistance genes find their way into wastewater. This has been demonstrated for extended spectrum β-lactamases (ESBLs) (Drieux, 2016) and carbapenemase producing Enterobacteriaceae (CPEs) (White, 2016). It is also clear that hospital waste contributes disproportionately to higher concentrations of CPEs which are predominantly human associated (Lamba, 2017). Wastewater treatment reduces the overall concentration of bacteria including antibiotic resistant bacteria (ARB) (Karkman, 2017), but some ARGs enjoy relative enrichment through the process (Mao, 2015). Since reduction of AMR in frequently applied wastewater treatment processes is limited, wastewater treatment plants (WWTPs) discharge ARB and ARGs in treated wastewater to the environment. Evidence of risk to humans from the use of human waste or even evidence of persistent ARBs in fertilized soils is scarce (Bondarczuk, 2016; Christou, 2017), but there is a need for more risk assessment of sewage sludge applications in agriculture. Studies have shown that fruits and vegetables, including those which may be eaten raw, can be contaminated with 3GC-resistant Gram-negative bacteria. WWTP effluent and agricultural run-off enter environmental waters where human exposure is possible via drinking water abstraction, crop irrigation/fertilization with wastewater or sewage sludge through the food chain (Manaia, 2016), or by direct contact with the environment due to occupational or recreational behaviours.

Concerns about the impact of wastewater on the environment and subsequent risk are escalated in rapidly developing countries, with less effective regulation of industrial effluent and less reliable treatment of wastewater. Where there is little or no treatment of wastewater, the environmental enrichment of ARB and ARGs by feces and sewage is a more serious issue (Fistarol, 2015). Multidrug-resistant *E. coli* strains are present in urban aquatic environments, even in countries where antibiotic consumption in both humans and animals is highly restricted, risk of environmental transmission must be assumed, even while it is not well quantified (Jorgensen, 2017). There is a concern about New Delhi metallo-beta-lactamase 1 (NDM-1) exposure through drinking water and/or water immersion in Southern Asia (Toleman, (2015).

Does the amount or type of resistant bacteria predict increased risk to human health?

The spread of ARB may result in human illness via such virulent waterborne pathogens as vibrio (Elmahdi, 2016; Poirel, 2005) and aeromonads (Lamy, 2009), involving primarily the consumption of

shellfish and direct exposure to recreational water, respectively. However, AMR-conferred threat to human health more commonly involves opportunistic human pathogens such as *Enterobacteriaceae* spp. and non-glucose fermenting gram-negative bacteria (e.g. *Pseudomonas*, *Acinetobacter* spp.). While AMR *Staphylococcus aureus* (MRSA) transmission may occur at the recreational water interface, it is less about environmental waters serving as an amplifying or disseminating reservoir and more about a 'venue' at which proximate human-to-environment-to-human transmission occurs (Griffith, 2016; Goodwin, 2012) , along with a potential role for marine mammals to serve as a source for human infection (Hower, 2013). In all cases, bacterial density will increase exposure risk and the probability of reaching an infective dose or a dose sufficient to result in colonization or in mobilization of ARGs to residents of the gut microbiome. The nature of exposure will also affect dose or number of ARB ingested, with head immersion water sports shown to result in much greater exposure than non-head immersion activities. For example, surfers ingest over 150 ml of water per session, whilst swimmers only ingest about 30 ml (Leonard, 2015).

Despite what may be high levels of ARB in environmental surface and sub-surface water, hygiene barriers can limit spread from these environmental sources into the four interfaces—and such hygiene barriers are more available in developed vs. under-developed settings (Walsh, 2011). For example, recreational water may be either treated to remove ARB or otherwise segregated from other environmental surface waters that are contaminated. Meanwhile, potable water finishing plants may use superior methods to remove and deliver ARB-free water at the tap, sewage may be kept from fisheries and bivalve sea beds, and only relatively uncontaminated water may be used to irrigate produce. Commonly, risk assessment and risk management frameworks are used to protect consumers, such as HACCP, bathing water profiles, and water safety plans. Such frameworks should be evaluated with respect to their value to avert AMR spread and emergence. Nonetheless, high levels of ARB contaminating environmental surface waters may result in gradients of relative contamination that make resisting the downward flow of ARB from environmental surface water to humans more difficult to overcome using hygienic barriers.

How does the interaction between bacteria and antibiotics impact AMR?

It is becoming clear that there is much more research needed to be done before we fully understand selection for AMR in environmental, animal, and human microbiomes. It also still remains to be determined what contribution to selection for AMR in the environment antibiotic residues make relative to other bioactive compounds, such as metals and biocides (Gaze, 2005; Gaze, 2011; Song,

2017). Traditional thinking defines the "selective window" where antibiotic resistance is favoured over susceptibility as the concentration range between the minimum inhibitory concentration (MIC) of a susceptible strain (MIC_{susc}) and the MIC of a resistant strain (MIC_{res}). However, there is increasing data showing that even below the MIC_{susc}, resistant strains maintain a relative advantage to survive over susceptible neighbours (Gullberg, 2011). The lowest concentration where this phenomenon is still observable has been termed the minimal selective concentration (MSC). If MSCs are above environmental antibiotic concentrations, then there is a potential for selection for AMR to occur. Efforts have been made to assess the MSC of a range of antibiotics in single species *in vitro* experiments, showing that the MSC can be significantly lower than the MIC, in some cases >100 times lower. For example, the MSC for ciprofloxacin, an important antibiotic for human health, was shown to be as low as 100 ng/L, which is well within the range of measured environmental concentrations. For other antibiotics, the MSC is significantly higher, and the ratio of MIC to MSC is dependent on resistance mechanism (Gullberg, 2011). Subsequent work by the same authors has shown that plasmid mediated resistance has a higher fitness cost and associated MSC than the same mechanism in a chromosomal location and that mixtures of antibiotics and antibiotics and other bioactive compounds such as metals can have additive or synergistic effect (Gullberg, 2014). Efforts have been made to estimate MSCs in complex microbial communities, giving improved ecological realism over the single species experiments. This was attempted for tetracycline in a freshwater biofilm, reporting an MSC of 1 µg/L (Lundstrom, 2016), although it is not clear if positive selection was observed or increased persistence (differential rate of negative selection). Further work has attempted to estimate the upper boundaries of MSCs based on MICs and then to apply an assessment factor to generate predicted no effect concentrations (PNECs) for a range of antibiotics, ranging from 8 ng/L to 64 µg/L (Bengtsson-Palme, 2016).

Future research needs

The following research topics on the impact of AMR in the environment on human health are recommended for prioritization:

- Existing hygiene, sanitation and other intervention measures and risk management frameworks to control infectious diseases should be evaluated for their efficiency in reducing the emergence and spread of AMR;
- Studies in travelers to new environmental (i.e. water and food) exposure settings—detailed food, water, and recreational exposure histories, microbiologic sampling of these environmental exposures, and assessment upon return for colonization with ARB and ARG are required;
- Additional ambient recreational water epidemiology studies evaluating gut colonization and skin, ear, and eye infections;

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- The risk posed by ARB bearing ARGs discharged into the marine environment should be assessed through longitudinal surveillance of resistance in human isolates of important marine pathogens such as *Vibrio* species;
- Full plasmid sequencing in wastewater microbial communities and in pathogens isolated from people may provide sufficiently granular analysis both to establish linkage and to infer directionality of transmission and may be an important target for research funding;
- Development of microbial risk assessment models to better understand AMR risks associated with various exposure routes and fecal sources (animal versus human sewage, point versus non-point) are required;
- Better understanding of selective potential of environmental residues of antibiotics and co-selecting compounds such as biocides and metals;
- Better understanding of the role phage plays in the transfer of resistance genes.

*Although other food for human consumption (i.e. meat and aquaculture fish) may also be directly contaminated through water use, because of the major risk of AMR determinants from the animal/aquaculture use of antibiotics, it is difficult if not impossible to distinguish the roles of animal (fecal) sources of AMR determinants from the environmental water source itself. Although AMR determinants in environmental water may also serve as the direct or indirect source of transmission to food-producing animals and aquaculture, and then via the food supply serve as source for humans, this review does not include such indirect causality.

Conclusion

With respect to risk assessment of the threat to human health posed by ARB and ARGs, it is important to remember that microbial communities are in a dynamic evolutionary state and that such a task is extremely complex (Ashbolt, 2013). A current absence of evidence or existing low risk should not reassure us about current or future risk. Absence of evidence is not the same as an absence of risk, and to a great extent reflects the small number of studies that have looked at the environment/human interface in the context of exposure, colonization, and infection by AMR pathogens. Environmental pollution containing bacteria, pharmaceutical, and personal care products are likely to pose a risk to human health in terms of opportunities for driving recruitment of novel ARGs to human and animal associated bacteria from the environmental resistome and in terms of acute exposure and infection risk to humans through environmental transmission pathways. Furthermore, continued natural selection in the presence of microbial and chemical pollution will almost certainly increase concentrations of ARB and ARGs over time, and one would expect a dose-response relationship with health risk. We must also consider that risks from wastewater could increase with climate change (Sterk, 2016).

C. Measurement

How should the presence of AR pathogens in the environment be measured?

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Methods for detecting and enumerating AR pathogens and genes

Many methods are available for the detection of AR pathogens and genes in environmental samples (Table 1). As these methods vary in sensitivity, cost, and technical requirements, different applications will be best served by different approaches. Advantages and limitations of each method are described below.

Table 1. Major methods for the detection of resistant pathogens and resistance genes.

Method	Target	Benefits	Limitations	Cost	Technical requirements
Laboratory culture	Pathogens	Quantitative; can have high sensitivity; detects phenotypic resistance; determines MIC	Limited to culturable organisms	Low	Low
Whole genome sequencing	Pathogens	Can detect all known resistance genes. Links resistance gene to host organism	Must culture organism first; cannot predict MIC	Medium	High
qPCR	Genes	Culture not required; quantitative	Limit of detections vary; limited number of targets; does not link gene to host organism	Medium	Medium
Metagenomics	Genes	Can detect all known resistance genes; culture not required	Limit of detection unknown; does not reliably link gene to host organism	High	High

Culture-Based Methods

Microbial culture, whereby microorganisms are grown and counted in the laboratory, has historically been the gold-standard approach to AR detection. Broth microdilution is the preferred method to determine whether an isolate is susceptible or resistant to a given drug; this is defined by the minimum inhibitory concentration (MIC) towards that drug. Standardized protocols, as well as cutoffs for assessing resistance or susceptibility, are available. MIC determination also allows monitoring of step-wise increases in resistance ('MIC creep') that may be missed with methods that return only susceptible/resistant determinations. However, MIC cutoffs to determine susceptibility are based on clinical treatment outcomes and may not be appropriate for environmental monitoring. The use of epidemiological cutoffs (ECOFFS) based on population MIC distributions or ecological cutoffs based on arithmetic MIC distributions have been suggested (Martinez, 2015). Alternatively, disk diffusion is a simpler method that can be used to determine resistance and estimate MICs, but it with less precision than broth microdilution.

Culture-based methods are the gold-standard approach to AR detection because they are inexpensive, quantitative, and easily transferred from clinical settings. Culture-based detection of antibiotic resistance in environmental samples uses a variety of selective or screening media to isolate the bacteria of interest. Commercially available media exist that target a wide variety of bacteria. Equipment requirements are minimal, making this approach well suited to low resource settings. In contrast to molecular methods, culture-based detection ensures that the bacteria detected are viable and meet regulatory cutoffs for resistance. AR bacteria can be isolated directly from samples by including antibiotics in the selective media, and, if parallel tests are conducted without antibiotics, this will allow estimation of the proportion of a bacterial community that is resistant.

However, culture-based approaches have substantial limitations for environmental microbiology. Most bacteria cannot be cultured in the lab, a limitation that is particularly profound in environmental samples (Hugenholz, 1998; Staley, 1985), and many bacteria can enter a state where the microbe is alive but does not multiply under environmental stress. For bacteria that can be cultured, the process can be time-consuming, requiring long incubations, multiple steps, and confirmatory analyses. Sample storage method and duration can strongly influence recovery and quantification of the target organisms. Perhaps the greatest limitation of culture-based methods is that they are not high

throughput. Drug-bug combinations to test for resistance must be assessed independently, and diversity measures are limited by the number of isolates selected for further characterization.

Molecular methods

Molecular methods are used to characterize bacterial isolates (commensals and pathogens), and to detect, enumerate and track antibiotic resistance genes from environmental samples (soil, water, manure, insects, etc.). Targets include antibiotic resistance genes themselves, as well as genes like integrase that are associated with the exchange of genes between bacteria.

The polymerase chain reaction (PCR) is the foundation for many molecular methods. Standard PCR methods are able to provide presence/absence information for a target gene, but do not provide information on what proportion of a sample is resistant (enumeration). If well designed, they are robust, economical, and easy to use (Storteboom, 2010). Quantitative PCR (qPCR) assays allow for enumeration of the target, but struggle with issues related to limit of detection (the lowest quantity of the target sequence that can be identified in a sample), particularly for environmental samples that have many inhibitors and low quantities of the target gene. Furthermore, qPCR methods are more expensive than standard PCR, and rely on comparison with a standard to enumerate, making it difficult to compare data between laboratories. However, the value of quantitative data for evaluating impact of management practices on AMR makes qPCR a common choice for studies evaluating AMR in a field setting (Graham, 2016; Tien, 2017). Commercial companies are using the qPCR platform for products designed to quantify multiple ARG targets simultaneously in 96- or 384-well formats (Agga, 2015; Karkman, 2016). As with individual qPCR assays, limit of quantification can be an issue, particularly because reactions cannot be individually optimized for each individual target. Alternatively, droplet Digital PCR (ddPCR) uses new technology to aerosolize a sample into thousands of individual droplets, which are individually assayed for ARGs using standard qPCR methods (Cave, 2016). It eliminates the limit of quantification issue of qPCR, and is more accurate than qPCR, but expense, complexity of assay development, and accessibility have limited its widespread use for measuring resistance in environmental samples to date.

A second set of molecular methods relies on DNA sequencing. In amplicon sequencing, a single gene – often the 16S rRNA gene, is amplified using PCR, and all of the resulting amplicons are sequenced – capturing the many varieties of the gene in the sample. Functional genes, such as antibiotic resistance genes (ARGs) can also be targeted. A second sequencing approach that incorporates an initial PCR step is

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epicPCR, which allows for sequencing whole communities in a way that links the 16S and AR genes for each cell, allowing attribution of the resistance to a specific bacterium. The method was designed to address questions in microbial ecology, and has been demonstrated to work in environmental samples (Spencer, 2016).

WGS and MALDI-ToF MS are molecular approaches to AR determination in bacterial isolates. WGS can be used to detect known AR genes in isolates and the predicted resistance has been shown to correlate well with phenotypic resistance in clinical isolates (McDermott, 2016; Tyson, 2016; Tyson, 2015; Zhao, 2016). WGS is now commonly used for public health AR surveillance efforts (McDermott, 2016), but its accuracy has not been evaluated for environmental bacteria. Currently, WGS is only able to determine whether resistance genes are present, and not the level of resistance, though methods to estimate MICs from WGS data are being developed (Nguyen, 2018). Moreover, WGS can only detect known resistance genes. WGS does provide information on genetic mobility of antibiotic resistant genes (ARGs), as well as ARGs that are genetically linked, which can be critical for determining the risk of horizontal transmission of ARGs. MALDI-ToF MS is a quick and reliable approach for bacterial identification, even for hard to culture organisms (Biswas, 2013). Test modifications have been developed to improve sensitivity and accuracy of MALDI-ToF MS, for example to detect AR phenotypes by detection of AR proteins, modification or breakdown of the target antibiotic, or inhibition of bacterial growth in the presence of antibiotics (Biswas, 2013; Choquet, 2018; De Carolis, 2017; Idelevich, 2017; Miltgen, 2018; Oviano, 2017).

Molecular methods are faster than culture-based methods, and can detect the presence of ARGs even in bacteria that are difficult to culture in the lab. A recent review highlights the strengths and weaknesses of molecular methods, specifically as they relate to measuring antibiotic resistance in environmental samples (Luby, 2016). Although presence of the target gene generally classifies a sample as having resistance, it is important to note that detection of the gene is not equivalent to resistance as defined by clinical standards (CLSI, 2018; Standards, 2006).

Metagenomics

Here, we reserve the term "metagenomic" to refer to high-throughput methods that sample all genes (or proteins) in a community without the need for laboratory culture. Broader uses of this term in the literature, for example to refer to community 16S sequencing for taxonomic purposes, are not included

here because they do not identify AMR genes or pathogens. Currently, there are at least three broad classes of metagenomic methods that can be used to detect AMR genes in environmental samples: classical metagenomics, meta-transcriptomics, and meta-proteomics.

In classical metagenomics, total DNA extracted from an environmental sample is sequenced extensively. Resistance genes in that environmental sample can be then be identified based on sequence similarity to known ARGs. This approach has been used to detect ARGs in a range of human- and animal-waste samples, including sewage and waste water (Bengtsson-Palme, 2016; Guo, 2017; Yang, 2014), hospital waste (Froes, 2016), animal and human faeces (Munk, 2017; Petersen, 2015), and in the guts of farm animals and humans (Auffret, 2017; Fitzpatrick, 2016; Thomas, 2017).

Similar in concept to the meta-genomic approach, ARGs can be inferred from the presence of their mRNA or protein products in a sample. "Meta-transcriptomics" refers to the deep sequencing of RNA molecules in a community sample, while "meta-proteomics" refers to high-throughput detection of proteins in an environmental sample by mass spectrometry. These approaches have the benefit of showing that ARGs are expressed in a sample, whereas classical metagenomic methods demonstrate only their presence. Furthermore, expression of ARGs may indicate that resistant cells are living in a sample, whereas classical metagenomic methods may detect DNA from dead cells. The use of these methods for ARG detection is still in its infancy, but proof-of-principle for meta-transcriptomics was provided by Versluis et al. who detected ARG expression in a variety of environmental datasets, including human and animal guts (Versluis, 2015). Rowe et al. compared meta-genomic and meta-transcriptomic analyses of ARGs in hospital effluents, and found close agreement between the two methods (Rowe, 2017).

The main benefit of metagenomic methods is the ability to detect many different ARGs in a sample – whereas PCR-based methods require a separate test for every ARG of interest. All ARGs present in a sample (above the limit of detection) can be detected in a single metagenomic sequencing run.

Several limitations remain for metagenomics: these methods are expensive, and quantification is limited to proportions rather than absolute numbers of resistant organisms. Sensitivity can be limited and may vary significantly, because reads from ARGs are only a small proportion of the total number of reads (Fitzpatrick, 2016; Vikram, 2017); targeted metagenomic approaches may help to address this issue (Lanza, 2018). Consistency between labs is an issue that needs to be addressed if metagenomics is

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to be used widely for surveillance, because variation in any step of the process from DNA extraction to data analysis can lead to different estimates of ARG abundance (Knudsen, 2016; Albertsen, 2015). Moreover, assigning a given ARG to a specific host organism is difficult, particularly for plasmid-borne genes (although cross-linking methods provide a possible solution), and this may be problematic for epidemiological investigations. Additionally, the level of taxonomic identification (i.e. family, genus, species, strain, etc.) for bacteria in the sample is limited by the sequence databases used for analysis. Reliable identification of bacterial species is not possible with the current databases (Bengtsson-Palme, 2017).

Functional genomics

Despite the benefits offered by meta-'omic strategies, they can only detect known resistance genes (or proteins). They will fail to detect novel resistance genes that have little resemblance to previously identified ARGs, and may misclassify genes that have acquired activity against new drugs (e.g., the acquisition of quinolone activity by aminoglycoside acetyl transferases (Robicsek, 2006)).

Novel ARGs can be identified using functional genomic approaches (reviewed in (Mullany, 2014; Dos Santos, 2017)). Here, fragments of genomic DNA from an environmental sample are cloned and expressed in a convenient host, typically E. coli. Transformed hosts can then be screened for resistance to an antibiotic of interest and the resistance gene identified by conventional sequencing. Functional genomic approaches have been used to identify novel ARGs in a wide variety of environments (Allen, 2009; Donato, 2010; Marathe, 2018; Sommer, 2009; Uyaguari, 2011).

While functional genomics is a powerful tool for identifying new ARGs, it is unlikely to be useful in general surveillance. The time and effort required to process a single sample is substantial, and the use of a single host species (e.g., E. coli) limits the number and type of ARGs that can be detected in a given experiment.

Do methods differ if testing for attribution (e.g., tracking resistant pathogens to a source like hospital, septic systems or farms)?

It is sometimes necessary to track a resistant pathogen, or a resistance gene, to a specific source, such as a hospital or a farm. Such epidemiological investigations require methods with a high degree of resolution – that is, the ability to distinguish between closely related genes or pathogens.

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WGS of bacterial isolates is the gold-standard for attribution – indeed, because the entire genome of each organism is sequenced, WGS represents the upper limit for detecting variation. Even in pathogens with little overall diversity, isolates can be grouped on the basis of a few shared sequence variants, making this a powerful epidemiological approach. WGS of foodborne pathogens is now routine for the US FDA and CDC and the Canadian CFIA, and is used regularly in epidemiological investigations of foodborne pathogens in North America (and Europe?). Similar methods could be readily applied to environmental samples, with the caveat that bacterial isolates are required for standard approaches.

Technical and/or financial considerations may limit the use of WGS in some situations, in which case other techniques may assist in attribution. Multi-locus sequence typing (MLST), for example, involves PCR amplification and sequencing of multiple genes from an isolate, and has a long history in molecular epidemiology [49]. Similarly, pulse-field gel electrophoresis (PFGE), whereby isolates are grouped based on patterns of DNA cleavage, can help to establish relationships between strains. MLST, PFGE, and other methods have lower resolutions than WGS, and thus may not allow for positive attribution. This is particularly a problem in bacterial species or serotypes that harbor low levels of sequence diversity.

Metagenomic data may be useful for attribution, particularly when a resistant organism is difficult to culture, or when a resistance gene rather than a particular pathogen is the focus of an investigation. The use of metagenomic data for attribution is subject to the limitations described above; recent studies suggest metagenomic data do have promise in epidemiology. Proper attribution and tracking for ARGs may require targeted sequencing of plasmids, which are often lost during metagenomics assembly.

Can these methods be standardized and used to monitor the impact of mitigation measure?

For culture-based methods, there are already well-formulated standard procedures for measuring antibiotic susceptibility. Culture-based methods are widely used to monitor the impact of mitigation measures in clinical and agricultural settings, such as the effects of antibiotic restriction protocols in animals and humans. Molecular typing of cultured isolates, such as MLST or WGS, is increasingly used to provide additional epidemiological data, and standardized methods are available for clinical use. The same approaches could readily be used to monitor the impact of mitigation methods in environmental samples. Culture-based methods are most appropriate when one or a few specific

bacterial species are to be monitored; generic E. coli are often used as an indicator organism for levels of resistance in the overall community.

In other cases, there may be an interest in monitoring the overall pool of ARGs and/or resistant organisms, necessitating the use of molecular or metagenomic methods. Currently, there are no widely-used standard procedures for the use of molecular or metagenomic methods in monitoring. PCR-based methods are readily standardized and very common in clinical diagnostics. However, there are no widely accepted PCR-based techniques for detecting ARGs in environmental samples, likely due to the difficulty in developing a method that will work in all (or many) matrices and a lack of consensus for which ARGs should be targeted. Metagenomic studies are highly sensitive to variations in protocols. Differences in DNA extraction technique, sequencing platform, and bioinformatic pipeline can have substantial effects on the outcomes of metagenomic analyses. Developing a standardized protocol for metagenomics analysis is challenging at this time due to limited validation of metagenomic methods and the rapidly changing technology. Further work on developing standardized qPCR and metagenomic pipelines, as well as reference materials, will aid in culture-independent monitoring.

D.   Mitigation

What mitigation methods are effective in preventing contamination of the environment or decreasing the amount of AR pathogens in environmental waters?

A myriad of mitigation options exists for preventing and-or reducing the amount of AR pathogens/bacteria in the environment, ranging from non-technical solutions that reduce/alter antibiotics use and "pollutant management" to high technology options, such as advanced oxidation and tertiary wastewater treatment. The same types of mitigation options apply to human and non-human mammalian (animal) wastes, although interventions and technologies used for animal waste streams tend to more rudimentary than those for human systems. This section primarily focuses on mitigation as it relates to human systems, partly because more information is available. However, technologies are similar to animal systems and to combat environmental AR, mitigation solutions must be holistic, following a One Health ethos that combines non-technical and technical solutions within both a human and animal context.

What are we mitigating against?

When considering mitigation methods, one must first define what one is mitigating against. The primary goal is to reduce human exposure to human AR pathogens as they directly impact less-treatable

infectious disease. However, one must also mitigate against AR commensals and environmental bacteria, and phage vectors that may carry and potentially share antibiotic resistance genes (ARGs) with pathogens, creating "new" AR pathogens of human health consequence. Evidence suggests that mitigation also should target ARGs themselves, either as free DNA, associated with phage, mobile genetic elements (MGEs), or within cultivable and uncultivable bacteria in the natural environment. However, debate exists about relative importance of ARGs in the environment alone as an explicit driver of AR pathogens. Recent work has shown horizontal gene transfer can occur between bacteria from environmental and hospital habitats with limited fitness costs [Chamosa 2017]. Although this potentially explains the evolution of acquired AR in some pathogens, it does not confirm rates or practical prevalence. Regardless, ARGs and MGEs clearly can indicate waste exposures and, in turn, the potential for AR pathogens in environmental reservoirs.

General mitigation approaches within a global context: Tiered solutions?

Mitigation methods for reducing the amount of AR pathogens/bacteria in the environment include non-technical and technical options, which range in applicability depending available resources and cultural context of the interventions. However, no single mitigation method has proven to be successful. Specifically, all evidence suggests that managerial interventions (e.g., use less antibiotics) without parallel technical interventions (e.g., enhanced waste treatment) or vice versa will not reduce environmental AR levels, especially in 80% of world where waste treatment functionally does not exist [Graham et al. 2014]. This statement holds true for both animal and human AR-related mitigation options, further suggesting a One Health approach is critical to global solutions. Specifically, growing evidence suggests that AR microbes can move rapidly across continents due to wastewater, tourism and trade [Zhu 2017]. As an example, class 1 integron abundances are increasing across the natural environment [Gillings 2015], a gene element that can enable bacteria to capture and transmit AR genes. However, genetic analysis suggests this gene may have arisen from a single cell in the early 20th century [Gillings 2014], implying the rate and scale of global spread.

Within this global context, possible mitigation methods are tiered, based on the expense and relative efficacy of each option, although limited information exists on the relative effectiveness of some options in terms of reducing AR pathogens, bacteria and-or genes in the environment (see later). Overall, more prudent antibiotic use is essential on global scales, but how improving use is best coupled to other interventions depending upon resources and context. General mitigation options include:

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1. Social, behavioural and managerial interventions, such as more prudent antibiotic use, but also the promotion of altered human or animal behaviour to reduce untreated fecal releases directly to the environment, such as open defecation [Ahammad 2014].

2. Parallel managerial interventions, such as reducing pollutant releases at source that might promote co- and cross-resistance in AR bacteria (reduce heavy metal and biocide releases to the environment) [Pal 2015]

3. Implementation or improvement of local wastewater management that spans:

    a. Provision or placement of toilets (even without treatment) in homes, communities and-or strategic locations to reduce open defecation;

    b. Provision of "local", decentralized wastewater management options that will delay fresh fecal matter entering enter receiving waters (e.g., portable toilets) or toilets connected to minimal local "treatment" (e.g., septic tanks, soak-ways);

    c. Provision of sewer collection systems that carry community and other wastewaters to a centralised treatment facility, which includes primary, secondary (biological), and-or tertiary treatment.

    d. Provision of sewer collection networks that include targeted pre-treatment for wastes from selected critical sources (e.g., hospitals, manufacturing facilities etc.), which would reduce the AR burden on central wastewater treatment systems.

    e. Provision of sewage collection and treatment networks, which also provide more stringent treatment and-or processing of wastewater biosolids.

    f. Provision of sewer collection systems with local pre-treatment and centralised community wastewater treatment, but then additional post-tertiary treatment that might ultimately allow for water reuse.

Many other variations exist and also combinations of the above with preferred mitigation options in any scenario being based on available resources. As an example, in "Least Developed Low-to-Middle-Income Countries (LMICs; OECD, 2018]", viable options for reducing AR pathogens/bacteria in the environment might be better control of antibiotic use combined with increased toilet access and improved rural and decentralised treatment. Conversely, in more developed countries where water reuse may be critical due to scarcity, layers of waste treatment may be needed, ranging for tertiary treatment options for

wastewater itself to advanced water treatment processing prior to reuse. Current options for reducing AR loads to the environment in most LMIC countries are limited and do not differ from typical management interventions in animal operations in the developed world. Therefore, much can be learned by co-examination of AR reduction methods across contexts to developed holistic solutions of global relevance.

Evidence of mitigation options for reducing AR pathogens/bacteria in the environment

There is growing data on the relative effectiveness of different mitigation methods for AR removal, especially for secondary and tertiary wastewater treatment. However, there also is considerable contradiction across the literature about the "best" options. Further, there are some migration methods, particularly more rudimentary options, such as septic tanks and other decentralised options, where almost no data exists on mitigation potential. Beyond this major knowledge gap, much information on AR mitigation is too observational, tending to overly emphasise antibiotic resistance gene (ARG) abundances (easy to quantify using methods such as quantitative Polymerase Chain Reaction; qPCR) rather than AR bacteria, including pathogens, which demands more labor and expense because of the wide array of potential targets. As such, details on removal mechanisms within most technologies are poorly understood, especially rates and extents of horizontal gene transfer in treatment processes and also within receiving waters. Finally, what happens to ARGs and AR bacteria within the biosolids stream is less studied, both in terms of biosolids processing technologies or AR fate in biosolids released to soils in the environment. This may be an important, but less understood, pathway for human exposure through food and releases from fields to receiving waters.

Within this context, what is known and-or can be achieved in different technical mitigation options as follows:

1. *Altered use and tighter antibiotics control* - Reducing antibiotic use clearly can reduce environmental AR. A good example is when Denmark stopped antibiotic and biocide use for growth promotion in agriculture in the 1990s, which resulted in significantly reduced AR bacterial [Aarestrup 2001] and ARG levels in previously impacted agricultural soils [Graham 2016]. However, data are less conclusive for most scenarios, which is a key knowledge gap regarding mitigation; i.e., how much less use is needed to reduce environmental AR levels and, in turn, human risk? Recent work by Singer's group suggested antibiotic use might need to drop by over 80% to reduce AR exposures in the environment to "non-risky" levels [Singer 2017]; a reduction is use that might compromise health care efficacy. Therefore, altered use must be

coupled with "smarter" waste treatment, including targeted treatment with particularly effective technologies at points of greatest antibiotic use and-or of ARG and AR bacteria release. Greater control over antibiotic use for non-therapeutic purposes also must be imposed at global levels as evidenced by recent reports of highly elevated carbapenem and colistin resistance due to colistin use as a food additive in Indian agriculture [Davies and Walsh 2018].

2. *Septic tanks, soak-away etc.* – There is almost no data on the mitigation value of sub-secondary treatment waste management technologies and there also is dearth of affordable and available small-scale treatment options. One example is denitrifying downflow hanging-sponge (DDHS) reactors that can reduce AR genes and bacteria by over 90% at small scales and for almost no energy cost [Jong et al. 2018]. However, this is only one example and there is a broad lack of available technologies, which is a major gap in mitigation. This is globally relevant because such "minimalist" technologies may be the only option for removing AR genes and bacteria from wastes in most of the world in the foreseeable future. For comparison, preliminary data hint that septic tanks can reduce ARGs and AR bacteria by up to 50% if they are well-maintained. Therefore, this may be the "most minimal" option. However, regular maintenance is not always provided, resulting in AR releases from septic tanks to the environment with functionally no treatment [e.g., Graham 2011]. It should be noted this is not just an emerging world problem, but also exists in the rural US and other developed countries [Wedgeworth and Brown 2013].

3. *Conventional secondary wastewater treatment* – Wastewater treatment plants (WWTPs) employ various treatment steps. Initial screening and primary sewage settling remove inert and biological solids, including AR bacteria within the readily settable solids. Removal versus passage of ARGs and AR bacteria after primary settling depends on the technology used in biological treatment step. Biological treatment is intended to remove soluble organic matter; i.e., microorganisms grow on that matter, including organisms from the original wastes and also organisms enriched in the process. After treatment, this mixed microbial community is separated by secondary settling (or sometimes by filtration) from the liquid stream, creating two effluent streams; i.e., supernatant liquid effluents and biosolids, which are processed separately (see section 6). Therefore, AR bacteria and ARGs go through different processing steps and, depending on each step, differential removal occurs of the AR bacteria [Al-Jassim 2015], ARGs [Christgen 2015] or other AR carriers, the remaining fractions being disseminated to the environment [Zhang 2017], either through the liquid and solid effluent stream.

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Biological treatment steps range from suspended floc (e.g., activated sludge) treatment to attached biofilm processes to mixed or more complex aerobic and anaerobic stages. Removal of bacteria in conventional biological treatment is around 90%, whereas the fate of specific AR bacteria and ARGs is more varied, depending upon the reactor type, oxidation-reduction conditions, the nature of the sewage and other factors. Up to about 99% removals can be achieved in secondary treatment for ARGs in liquid effluent, although this does not account for ARGs and AR bacteria separated into the biosolids stream. In general, there is concern that influent bacteria, including pathogens, and ARGs in the presence of residual metals and antibiotics in the wastes, might promote elevated horizontal gene transfer ARGs within biological treatment systems. Although there is some evidence this occurs, rates of gene transfer in activated sludge appear to be relatively low [Munck 2015], although much more work is needed to confirm too what extent and between whom gene transfer occurs in treatment processes. This knowledge is needed to make WWTPs as effective as possible at reducing AR bacteria and genes from wastes.

Although debate exists about the importance of gene transfer, ARG fate and AR bacterial selection in treatment processes, such processes are essential to human and environmental health. Growing evidence suggests a major reason why AMR is increasing on global scales is due to the wide lack of secondary level treatment in most of the world (rather than weaknesses in existing technologies). This does not mean current biological treatment options are perfect because there is evidence specific types of resistance can be preferentially selected, such as multi-drug resistant phenotypes and genotypes [Czekalski et al. 2012; Yang et al. 2013]. There also is strong evidence that a small sub-fraction of AR enteric bacteria that enter WWTPs in the wastes, including pathogens, selectively survive our current secondary treatment systems (Quintela-Baluja 2018). However, underpinning reasons for this require further investigation. Process modifications and retrofits of existing WWTPs are being developed to address these weaknesses to improve the ability of existing WWTPs to reduce ARB and ARG releases. For example, sequencing anaerobic-aerobic bioreactors have been shown to reduce ARG diversity and abundances in treated effluents by 60% [Christgen 2015]. Further, membrane-separation technologies (MBRs) have shown particular promise at ARG and AR bacteria removal [Harb 2016], and source pre-treatment prior to release to sewers might also be effective at removing organisms particularly capable of horizontal gene transfer prior to entering WWTPs (e.g., see section on hospital emissions).

4. *Tertiary wastewater treatment* - Tertiary treatment options for secondary WWTP effluents include the use of disinfectants and other oxidants and various filtration or membrane options. Chlorine disinfection can achieve approximately 99% removal of bacteria (also assuming AR bacteria) using typical chlorine doses and contact times However, AR bacteria appear slightly less susceptible to chlorination and higher doses may be needed [Munir 2011]. Although higher doses may improve AR bacteria reduction, such doses may generate higher levels of potentially carcinogenic disinfection by-products [Garner 2016; Zhang 2017], which is a particular concern for potential water reuse.

    Ultraviolet (UV) disinfection is an alternative to chlorine because it does not generate disinfection by-products. Doses between 5.0 and ~200 mJ/cm^2 are typically used to inactivate microbes in normal disinfection and doses between 10 to 20 mJ/cm^2 have be found to inactivate over 99.9% of the AR bacteria [McKinney and Pruden, 2012]. However, ARGs appear less susceptible to UV with only 90 to 99% removals being observed at comparatively higher UV doses. This sounds promising, but UV systems are less effective in the presence of greater solid matter, a common problem with wastewater, suggesting the technology may be less effective or unreliable for reducing ARGs and AR bacteria in WWTP effluents.

    Beyond UV and chlorination, tertiary options include ozonation, other advanced oxidation processes (i.e., AOPs), nanofiltration and reverse osmosis for reducing bacterial and other loads. Ozone is a strong oxidizing agent, which has shown promise in increasing bacteria and pathogen destruction, which in turn, can impact many AR bacteria and ARGs with adequate doses and contact times [Luddeke 2015]. However, evidence suggests some strains can increase with ozonation, including AR *E. coli* and *Staphylococcus* spp. Despite these issues, ozonation is being promoted as a possible tertiary treatment option as it appears to be better than chlorination or UV, although it is very costly.

    Other tertiary mitigation options exist, including combining disinfectants and-or other technologies, such as microfiltration, ultrafiltration, nanofiltration and reverse osmosis. Of these options, membrane-based technologies seem most effective at reducing ARGs and AR bacteria. Such technologies can be used in tertiary wastewater treatment or possibly in water reuse, and can be effective against an array of bacteria. However, specific log-reduction data are rather limited for AR bacteria and ARGs, except with MBRs. Further, membrane-based mitigation technologies tend to be more expensive and would be limited to only well-resourced applications.

5. *Pre-treatment at source prior to entering the sewer system* – Some wastewater sources to sewers can have higher AR gene and-or bacteria abundances, or release AR bacteria that more susceptible to horizontal gene transfer. There also are sources that may have particularly high levels of biocides, heavy metals and other agents that can promote co-resistance. Although it is not currently practiced for AR, targeted treatment at source may be a valuable strategy for reducing the AR burden on existing community WWTPs. In fact, this may a preferred strategy because the expense of treating wastes is a function of the technology employed and the volume treatment. Source treatment is attractive because treated volumes can be much lower, which means more aggressive and costly technologies might be employed major AR sources. An example is provided in the section on hospital wastes relative to source treatment of hospital wastewater.

However, the case for source treatment is based on a greater understanding of sources that might be specifically targeted. Therefore, in order for a case to be made for source treatment, a better understanding of the nature of AR in different sources is needed. Hospitals are one option, but more data are needed to confirm this case. Targeting waste sources with higher heavy metals also is an option, but in both cases, more reconnaissance data are needed among sources. Such data includes AR genes, types, and bacterial abundances; potentials for gene transfer and co-selection; and other traits that might justify targeted pre-treatment of specific sources. If key sources can be identified, cost-effective pre-treatment solutions are possible that can be coupled with retrofitting existing WWTPs to reduce AR pathogens/bacteria released to the environment.

6. *Wastewater biosolids processing, including animal manure* – Research has shown that 90-95% of ARGs in untreated municipal wastewater are simply physically removed such that they ultimately reside with the wastewater solids [Munir 2011]. Numerous technologies are available and used, in practice, to stabilize (i.e., reduce the organic content) and to inactivate pathogens in residual wastewater solids. Not surprisingly, these technologies are also capable of reducing the quantities of ARGs with varying degrees of efficacy. In general, technologies designated by the US EPA as a Process to Significantly Reduce Pathogens achieve moderate destruction of ARGs, whereas Processes to Further Reduce Pathogens achieve more rapid and extensive destruction of ARGs (for examples, see https://www.epa.gov/biosolids/examples-equivalent-processes-pfrp-and-psrp). These same technologies can also be used to treat animal manure as well, although this practice is substantially less common. Instead, animal manure is usually

applied directly (with no or minimal treatment) to soils where ARGs decay at much slower rates. In fact, ARGs are still detectable at levels greater than pre-manure application for at least six months [Marti et al. 2014], which suggests that long-term accumulation of ARGs is possible in situations in which animal manure is applied to soil more than twice per year.

Treated wastewater solids are also applied to soils following treatment. The rates of ARG decay in soil appear to be similar to that of animal manure [Burch 2014; Sandberg and LaPara, 2016], which are much slower than that the above mentioned US EPA pathogen reduction processes [Burch 2013a and b]. The technology used to treat wastewater solids prior to application to soils is also important to the relative ARG fate in the soils. ARG levels in soils returned to background levels within six months when wastewater solids are treated using Processes to Further Reduce Pathogens, but ARG levels remain elevated compared to controls when wastewater solids are only treated using Processes to Significantly Reduce Pathogens [Burch 2017].
The treatment and handling of wastewater solids and animal manure offers a substantial opportunity for mitigating the spread of ARGs. This improvement can be achieved by more widespread implementation of the technologies most effective for treating wastewater solids (PFRPs), more widespread treatment of animal manure, and less frequent application of wastewater solids and animal manure to soils.

Are methods lacking or do we need to use what we have better?

There are no infallible mitigation options, but different options have different relative merit. Overall, optimal mitigation is the reduction of antibiotic use coupled with most feasible and effective treatment option possible given local factors However, much is to be learned about mitigation options. For example, fundamental research is needed to better understand our existing technologies, such that they can retain current effectiveness in removing carbon and nutrients, but not promote multi-AR selection (as an example) or use the copious amount of energy, which is typical of most secondary biological and tertiary waste treatment technologies. Furthermore, "new" more sustainable treatment options are needed for the wider world, which should be developed from what has been learned in developed-world applications. There has been recent progress in our understanding of horizontal gene transfer, ARGs and AB bacteria within current wastewater treatment systems, but much more knowledge is needed, especially identifying how existing WWTPs might be economically modified to reduce the potential of AR pathogen spread through treatment processes and within the downstream natural environment.

Tertiary treatment is currently the least feasible mitigation option due to its high cost. Ozonation might be capable of further reducing ARGs and AR bacteria in WWTP effluents, but at great expense due to massive liquid volumes treated at WWTPs. However, if one can track key sources of AR bacteria and ARGs among the waste network, technologies like ozonation might become cost effective because treated volumes at source will be much less. This strategy demands we develop a much better understanding of which sources are "worst", implying greater reconnaissance is needed now to identify whether and which sources should be targeted for specific treatment.

Mitigation in Practice: Should we treat healthcare facility wastewater prior to release into sewers?

Whether healthcare facility wastewaters should be treated prior to release to sewers is under debate. Lamba et al. (2017) studied CRE, bla_{NDM-1}, HGT and fecal indicators in wastewaters from Indian hospitals that had their own WWTPs. Very high levels of CRE and bla_{NDM-1} were found in the treated hospital effluents; however, qualitative evidence suggests that few of the WWTPs in the study were well managed or were suitable for reducing AR bacteria. Conversely, a recent Dutch study indicate untreated hospital releases could not be unambiguously linked to increased levels of CRE in mixed wastewaters (Schmitt 2017). The Dutch study also concluded it was difficult to distinguish between the effects of hospital wastewaters themselves and the impact of WWTP size in sewer catchments with and without hospitals.

Given hospital wastes typically contribute between 0.2 and 2% by volume of wastewater in typical sewers (Carrero 2015), there is debate about whether separate treatment of the healthcare fraction is a priority. However, Brechet et al. (2014) estimated that one-third of all extended-spectrum beta-lactamase-producing *E. coli* found in domestic wastewater was of hospital origin, which is concerning because this is from a small fraction of the total waste volume. Further, if AR bacteria from hospitals have greater MICs, MDR and potentially more prone to HGT, a case for treating hospital wastes at source can be made.

From an engineering and economic perspective the treatment of healthcare facility wastes might make practical sense. The cost of wastewater treatment is a function of the technology employed and the volume of waste treated. Therefore, if one treats less volume, more costly treatment options can become affordable. For example, ozonation is a potentially effective option for reducing AR bacteria and genes in wastewater (see mitigation section), but it is it expensive. However, if ozonation or other treatment options (e.g. membrane bioreactors) were used for healthcare wastes, the healthcare facility AR load in the sewers would be reduced. This would reduce pressure on existing community WWTPs to

remove AR bacteria and genes. The ultimate cost-benefit of pre-treatment depends on the "relative risk" with and without pre-treatment, which requires knowledge gaps be resolved to make informed decisions.

Conclusion

Mitigation interventions will be most effective by adhering to the following general guidelines:

1) Effective mitigation needs to be approached from a global perspective given that the risk from novel AR pathogens transcend geopolitical boundaries, independent of the source of origination;

2) Implementation of primary treatment in much of world without basic sanitation (in concert with reduced antibiotic use) should be emphasized;

3) Low-cost treatment options should be developed based on improved basic knowledge of the dynamics between AR genes and horizontal gene trasfer within treatment options; and

4) Intensive (and higher cost) treatment, if considered, must be focus on "high risk" waste sources.

III.   LITERATURE REVIEW

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<div align="center">**Antibiotic Manufacturing Waste**</div>

I.   BACKGROUND STATEMENT

Manufacturing antibiotics results in the release of antibiotics into the environment unless effective control measures are implemented. Without control measures, the amount of antibiotics released can be very high and result in increased levels of resistant bacteria in the environment. There is potential for this environmental contamination to affect human health and measures should be taken to minimize this risk.

Responding to this risk will require knowledge of manufacturing measures that minimize or eliminate environmental contamination from drug or drug compounds, standardized methods to monitor drugs or drug compounds in the environment, and agreement on acceptable discharges.

II.   SCIENTIFIC ISSUES

A. To what extent is the environment currently being contaminated with antibiotics from manufacturing waste?

The way antibiotics are produced, the resulting by-products, and the impact of factory effluents are issues often been neglected in AMR discussions. There is growing evidence of antibiotic manufacturers that do not adequately treat waste products, leading to the disposal of high concentrations of active antibiotic ingredients into the local environment, with the potential to select for and promote the evolution of antibiotic resistance (AMR Review, May 2016).

Manufacturing antibiotics

Around 10,000 different antibiotics are known, and 200-300 new ones are being added each year (Wohlleben et al., 2016). Most of these antibiotics are not commercially important due to toxicity, ineffectiveness, or high production cost. Approximately 120 antibiotic types are produced by industrial fermentation, while more than 50 semi-synthetic and many synthetic compounds (Figure 1) also have

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clinical applications as antibiotics (Lengeler et al. 1999). Of these products, there are approximately 50 antibiotics which are most widely used.

Antibiotic-producing microorganisms are grown in large vats, generally in quantities of 100,000–150,000 litres of liquid growth medium. By controlling oxygen concentration, temperature, pH, and nutrient levels, the manufacturer can ensure that maximum yields are produced by maintaining ideal levels of microorganisms. Once fermentation is complete, the antibiotic is extracted and purified to a crystalline product. This is easier to achieve if the antibiotic is soluble in organic solvent. Otherwise it must first be removed by ion exchange, adsorption, or chemical precipitation.

- Fermentation

Antibiotics are made completely synthetically in the lab. These include the quinolone class of antibiotics, of which nalidixic acid is often credited as the first to be discovered.

- Synthetic

•Antibiotics can be produced in a combination of natural fermentation and laboratory work to maximise production. Maximisation can occur through efficacy of the drug itself, amount of antibiotics produced, and potency of the antibiotic being produced. Depending on the drug being produced and the ultimate usage of said antibiotic determines what one is attempting to produce.

- Semi-synthetic

Figure 1. Main methods for production of antibiotics

In the earliest years of antibiotic discovery, antibiotics were naturally produced either by fungi (i.e., pencillin), or by soil bacteria (i.e., streptomycin and tetracycline (Clardy et al. 2009). Microorganisms used in fermentation are often genetically modified for maximum antibiotic yields. Mutation, using ultraviolet radiation, x-rays, or certain chemicals is often used, and reproduction of select, higher-yield strains can increase yields by 20-fold or more. Another technique used to increase yields is gene amplification, where copies of genes coding for enzymes involved in the antibiotic production can be inserted back into a cell, via vectors such as plasmids. This process must be closely linked with retesting of antibiotic production.

There are many ways that antibiotics can enter the environment, for example, as emissions, wastewater, or solid waste, dependent upon the stage at which storage tanks are cleaned in between production runs (Guardabassi et al. 1998). During the production of 1000kg of antibiotic (procaine penicillin G), 10,000kg of wet mycelium, 35,000kg of wet biological sludge, 56,000 litres of waste fermentation broth, and 1,200 litres waste solvents can also be produced (US EPA, 1976). The environmental effect of the pharmaceutical industry is more notable than that of hospital waste and general sewage, mainly due to the quantities of antibiotics involved in the production process.

Global production

The industry's supply chain for established antibiotics is complex and global, with many stakeholders involved (Figure 2). Antibiotic production is highly commercialised due to a heavy demand worldwide, and government authorities play a predominant role in regulating production.
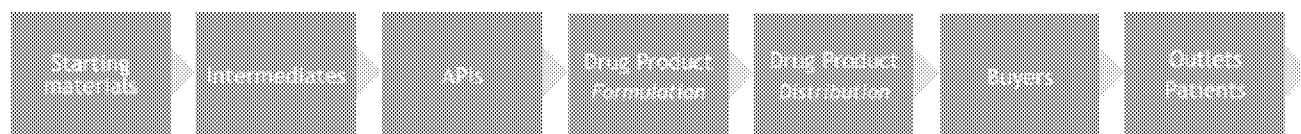

Figure 2. Antibiotics supply chain: a complex issue

It is estimated that annual worldwide antibiotic production exceeds 100,000 tons (Bbosa and Mwebaza 2013). By 2030, production is expected to increase by at least two-thirds to address increases in consumption due to increases in animal antibiotic treatments and the shift from extensive to intensive farming (van Boeckel et al. 2015). Asia is the world's main producer and supplier of active pharmaceutical ingredients, including antibiotics. Active pharmaceutical ingredients (APIs) are the biologically active substances within medicines that have an effect on the patient (human or animal). The Review on Antimicrobial Resistance (2016)[1] has concluded that many pharmaceutical producers, attracted by cheaper labour and capital costs and weaker environmental protection laws, have outsourced their manufacturing outside of Europe to India and China. China is the world's largest producer and exporter of APIs, currently supplying up to 90% of all antibiotic APIs. The majority of these are then processed in India before being sold to markets worldwide.

In comparison, annual production in the U.S. exceeds 16 million kg, around 16% of global production, an increase from 1954 when just under1 million kg of antimicrobials where produced annually in the U.S. (Huttner et al. 2013). Currently, an estimated 23×10^6 kg of antibiotics are used annually. Approximately half are provided to people, and the rest are manufactured for agriculture. Globally, livestock consumed

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[1] The Review on Antimicrobial Resistance (AMR), was commissioned in July 2014 by the UK Prime Minister, who asked economist Jim O'Neill to analyse the global problem of rising drug resistance and propose concrete actions to tackle it internationally. It was jointly supported by the UK Government and Welcome Trust.

at least 63,200 tons of antibiotics in 2010, accounting for nearly 66% of the estimated 100,000 tons of antibiotics produced annually worldwide. Consumption is projected to rise to 105,600 tons by 2030 (Third World Resurgence, 2015).

The link to Antimicrobial Resistance (AMR)

AMR is primarily caused by inappropriate use and overuse of antibiotics in humans and animals, but, increasingly, evidence shows that pharmaceutical waste from excretion and disposal (including pharmaceutical manufacturing effluent) is also a concern in the development of resistance (Küster and Adler, 2014; Ashbolt et al. 2013).

High concentrations of antibiotics in the environment can result from manufacturing emissions. This creates an unparalleled environment for the evolution of antibiotic resistance, which can spread through the environment through horizontal gene transfer, a process by which bacteria can share DNA between species (Kritstiansson et al. 2011, Flach et al. 2015). Evaluating this risk is vital, but with the current state of manufacturing companies not reporting their outputs and the overall lack of regulations, this is proving to be difficult (Larsson et al. 2007; Larsson & Fick, 2009).

A few reports have traced AMR to pollution from factories in India and China, noting that discharges of antimicrobials and their by-products are from various sources, including pharmaceutical production plants. These reports are raising concern for the potential development and spread of AMR in the environment.

In 2013 alone, Chinese mainland reportedly consumed almost 162,000 tonnes of antibiotics (almost evenly divided by human and animals). The research published by the Chinese Academy of Sciences showed levels of antibiotics in all of the country's 58 river basins for 36 commonly used antibiotics. Seven of the 36 antibiotics were were found at concentrations over 1,000 nanograms per litre (Zhang et al. 2015). China lacks environmental antibiotic concentration standards, but levels of 1,000 nanograms per litre were considered high. The waterways of Beijing, Tianjin, and Hebei were listed as the top three most affected provinces. Amoxicillin and Florfenicol were among the 36 antibiotics tested, with estimated 97,200 tonnes consumed and 53,800 tonnes ending up in the environment in the form of wastewater treatments and urine (Zhang et al. 2015). Similarly, in India, a treatment plant receiving wastewater from

multiple bulk drug manufacturers was shown to be a reservoir for highly multi-drug resistant integron-bearing bacteria (Marathe et a. 2013).

The lack of data

Currently there is little published information available concerning global quantities of APIs produced annually and where they are produced. The lack of data and transparency on these issues is cause for concern. Low pharmaceutical prices could be indicative of low manufacturing standards, resulting in environmental pollution. Problematically, current legislation fails to properly address this issue. The European Medicines Agency's "Guideline on the environmental risk assessment of medicinal products for human use" (2006) states that before receiving market authorisation, pharmaceutical products should undergo an environmental risk assessment. This requirement does not apply, however, to antimicrobials placed on the market before 2006 when the guidelines came into force, and no risk assessments on the development of AMR in the environment are required. There is no scientific evidence that products placed on the market before 2006 are of less environmental concern than new products.

Although there is a clear link between manufacturing and elevated levels of antibiotics in the environment, the lack of emission data makes it difficult to know the extent of the problem at every site. Companies do not report discharge levels voluntarily, and regulatory agencies do not collect such data or set limits. From the very few cases investigated, the high levels of antibiotics (Larsson et al 2007; Fick et al, 2009) and the high levels of resistance genes reported (Marathe et al. 2013; Johnning et al. 2013; Flach et al. 2015; Kristiansson et al. 2011; Bengtsson-Palme et al. 2014) should raise alarm over antibiotic manufacturing emissions and their role in the selection and spreadof AMR.

The need for wastewater treatment

It is clear that in terms of mass flow of APIs into the environment, the most common way antibiotics get into the environment is from the excretion of drugs that people used for therapy (i.e., human waste). The human waste goes through a wastewater treatment process, then any antibiotic residue (and resistance) remains in surface waters. However, in terms of impact and potential risks, the localized emissions from manufacturing plants may potentially be more important. Concentrations of APIs entering wastewater treatment systems are generally low because they are being used by only a small fraction of the population served. These loadings are further reduced by removal processes during treatment, albeit to

a variable extent. As a result, APIs are typically determined in effluents and receiving river waters at ng/L concentrations. However, in many low- and middle-income countries, the availability of modern wastewater treatment infrastructure is limited and can often be exacerbated by lower water availability or usage, which results the ability to dilute.

The direct API discharge of manufacturing plants can be significantly larger than emissions (Larsson 2014). Although there may only be a relatively small number of such facilities, reported concentrations in effluents are often orders of magnitude higher. For example, Larsson et al (2007) analysed a range of APIs in the effluent from a wastewater treatment plant serving about 90 bulk drug manufacturers in India and reported concentrations of ciprofloxacin between 28 and 31 mg/Land concentrations of fluoroquinalones between 0.15 and 0.9 mg/L. Lübbert et al. (2017) reported concentrations of moxifloxacin, voriconazole and fluconazole of 0.69, 2.5 and 240 mg/L, respectively, around a manufacturing site in India. Li et al (2008) also reported a concentration of 20 mg/L of oxytetracycline in treated effluent from a pharmaceutical manufacturing facility in Hebei Province, China. These elevated concentrations of APIs are not just limited to manufacturing effluents and river waters. For example, Kristiansson et al. (2011) reported ciprofloxacin concentrations of 914 mg/kg (OM) in sediment downstream of an Indian wastewater treatment plant. Although many studies have reported elevated concentrations of antibiotics in effluent streams in India and China, similar reports from around the globe have been reported (Larsson 2014). For example, Khan et al. (2013) reported concentrations of a range of antibiotics downstream of formulation facilities in Lahore in the µ/Lrange, up to 49 µ/Lof sulfamethoxazole. Sim et al. (2011) reported concentrations of up to 44 mg/L of lincomycin in the effluent from a pharmaceutical manufacturer wastewater treatment plant in Korea, and Bielen et al (2017) reported concentrations up to 3.8 mg/L of azithromycin in effluent from a Croatian pharmaceutical manufacturing plant.

More importantly, these apparently uncontrolled emissions from manufacturing plants have been associated with negative impacts on aquatic ecosystems and with antimicrobial resistance. The impact of such discharges to the wider environment and potentially to humans is an area that requires further study. The risks associated with the discharge of large quantities of APIs at a relatively small number of locations, compared to the widespread discharge of larger quantities of APIs via a vast number of largely treated point sources, are different and require further investigation. It is clear, however, that resistant pathogenic microorganisms need only develop at one site and widespread distribution is inevitable (Larsson, 2014).

Scientific gaps. Identification and application of appropriate treatment methodologies, possible remediation

A number of studies have attempted to assess the risks associated with the discharging manufacturing wastes largely to the aquatic environment. Risks associated with the production and disposal of solid wastes are largely unknown. There are a number of methods to reduce emissions from manufacturing wastes, which also reduces risks associated with API discharge. Improvements in manufacturing processes to increase production efficiency and reduce wastes have obvious benefits. Alternatives include the capture and treatment wastes, such as batch reactor washings before discharge. Standard wastewater treatment technologies have some ability to treat or remove APIs, but removal rates can vary. Such techniques may require innovation and improvement to make them more efficient.. Attempting to increase degradation requires careful monitoring of conditions and understanding what potentially harmful by-products may form in the process.. APIs could be removed by sorption or sedimentation, associating them with solids for removal. This would create additional solid wastes that might require special techniques for disposal. For any of these methods to be successful, oversight is required to ensure discharges are controlled and that there is consent to dispose of treated waste to the receiving environment.

Recommendations for addressing those gaps. Development of appropriate emission standards for effluents from manufacturing sites e.g. environmental reference concentrations (ERCs)

The EU Water Framework Directive currently includes diclofenac, ethinyloestradiol and oestradiol on its watch list of possible emerging contaminants, but otherwise there are no current regulations covering the discharge of APIs. However, the control of emissions of APIs from point sources, such as manufacturing sites and wastewater treatment plants, is technically feasible, although with cost implications. Emission limits could be developed with technical solutions to ensure compliance, assessed by a regulatory monitoring system, including enforcement and potential sanctions. Bengtsson and Larsson (2016) developed an approach to calculate upper-bound selective concentrations for antibiotics to aid the development of effective antibiotic resistance regulation. The study used minimal inhibitory concentrations (MIC) data for a wide range of antibiotics to calculate predicted no-effect concentrations (PNECs) for resistance, with resulting concentrations ranging from 8 ng/L to 64 µg/L. These values are

orders of magnitude lower than many studies reporting concentrations of antibiotics in effluents from manufacturing sites in India and China. Le Page et al (2017) also developed a risk-based approach to develop discharge targets from manufacturing sites. They provided a review and meta-analysis of antibiotic aquatic toxicity and minimum selection concentration data for clinically-relevant bacteria. They concluded that the current approach to environmental risk assessment (based on one cyanobacteria species) is insufficient and further data is needed on the effects of antibiotics on bacterial diversity, community structure, and ecosystem function. Based on the few data that are available, it is challenging to derive production discharge limits, but a conservative limit of 154 ng/L is suggested based on data from 27 antibiotics and no observed effect concentration (NOEC) data for a range of sensitive phyla. Based on this review of existing data, the authors suggest an antibiotic discharge threshold limit of 100 ng/L would be protective of environmental bacterial populations.

Supply chain management

There is likely to be increasing pressure applied to European and U.S. pharmaceutical companies to ensure their API supply chains comply with Good Manufacturing Practise. This compliance will also raise awareness to the major API suppliers in China and India of the importance of reducing the impact of their manufacturing processes in order to ensure their future markets. There are also increasing pressures being placed on manufacturers by their respective governments to reduce impact on local, regional, and global environments. The recent Davos "Declaration on Combating Antimicrobial Resistance" was signed by over 100 companies in 2016, requiring its signatories to "support measures to reduce environmental pollution from antibiotics". Another example of supply chain action is the Industry Roadmap for Progress on Combating Antimicrobial Resistance, published in 2016 by a dozen companies, including many of the largest research-oriented ones. Signatories agreed to a plan to reduce the environmental impact from production of antibiotics by:

> 1. Reviewing our manufacturing and supply chains to assess good practice
> 2. Establishing a common framework for managing antibiotic discharge, buildinging on existing work such as the Pharmaceutical Supply Chain Initiative (PSCI), and starting to apply it across manufacturing and supply chains by 2018.
> 3. Working with stakeholders to develop a practical mechanism to transparently demonstrate that supply chains meet the standards in the framework.

4. Working with independent technical experts to establish science-driven, risk-based targets for discharge concentrations for antibiotics and good practice methods to reduce environmental impact of manufacturing discharges by 2020.

B. Does environmental contamination result in an increase in AMR bacteria within the environment? If yes, to what extent does this pose a risk to human health?

Environments with no antibiotic contamination

Most antibiotics created and used were isolated from naturally-occurring soil fungi and bacteria (Waksman, 1940; Martin, 1989), and the antibiotic-producing strains of these fungi and bacteria carry natural resistance mechanisms to the compounds they produce (Hopwood, 2007). Evidence indicates that antibiotic resistance is extremely widespread in natural environments, including relatively pristine habitats with no direct exposure to anthropogenic activities (D'Costa, 2006; Brown, 2009). Lima-Bittencourt *et al.* (Lima-Bittencourt, 2007) found that more than 60% of the Enterobacteriaceae isolates from pristine freshwater environments were resistant to multiple antibiotics, while Brown and Balkwill (Brown , 2009) screened isolates from sub-surface environments (170–250m below surface) and found that more than 70% of the bacteria were resistant to multiple antibiotics. Perhaps most revealing, D'Costa *et al.* (D'Costa, 2011) analyzed ancient DNA extracted from 30,000-year old permafrost sediments and identified a diverse collection of genes encoding resistance to beta-lactam, tetracycline, and glycopeptide antibiotics.

Environments with low-level antibiotic contamination

It is estimated that up to 75% of antibiotics are excreted unaltered after they are used by humans or animals for the control of human disease and/or animal husbandry.(Böckelmann, 2009). Unfortunately, most wastewater treatment plants (WWTPs) are not designed for the removal of these micropollutants (Miao, 2004; Göbel, 2005) and, as a result, residual antibiotics are released into the environment with treated wastewater (Jury, 2011). Since the late 1990s, many classes of antibiotics have been reported in raw sewage and treated wastewater, including beta-lactams (Cha, 2006), sulfonamides, trimethoprim, macrolides (Göbel , 2007), fluoroquinolones (Lindberg, 2005), and tetracyclines (Karthikeyan, 2006). Antibiotics can be detected in natural water sources that receive treated wastewater (Kolpin, 2002; Yang, 2003), leading to increasing concern regarding the contribution of water reclamation to antibiotic resistance

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in populations of pathogenic and nonpathogenic environmental microorganisms (Pauwels, 2006; Auerbach, 2007). Examination of antibiotic resistance genes in river sediments contaminated by low levels of tetracycline and sulfonamides due to urban and agricultural activities in the United States showed significantly higher antibiotic resistance gene concentrations at the impacted sites compared to pristine sites where no antibiotics were detected (Pei, 2006). However, there was no statistically significant correlation between the concentration of antibiotics in the sediments and the concentration of resistance genes. It is not clear whether the elevated levels of resistance genes in the river sediments were a result of antibiotic selection taking place in the sediments, or if selection occurred upstream and the resistant microbes transported. Moreover, recent work has reported preferential elimination of non-resistant subsets of bacteria during wastewater treatment (Figueira, 2011), suggesting that that the preferential elimination of susceptible organisms rather than their resistant counterparts contribute to increasing resistance.

Recent evidence has shown that sub-therapeutic antibiotic concentrations can enrich for resistant bacteria (Silver, 1996), suggesting that if low-level concentrations of antibiotics are discharged into the environment, this may cause soil bacteria to develop resistance (Bengtsson-Palme, 2016). The presence of low-level antibiotics in wastewater used for irrigation or recharge would support the notion that resistance could develop in environmental bacterial in those receiving basins (Bengtsson-Palme, 2016; Gullberg, 2011; Andersen , 1996).

Environments with high-level antibiotic contamination

Effluents from antibiotic manufacturing units and the environments contaminated by such effluents in India, China, and Croatia contain high antibiotic concentrations (Galvin, 2010; Lübbert, 2017; Larsson, 2007; Li, 2009; Bielen, 2017; Li, 2008). For some antibiotics, the concentrations were much higher in these effluents when compared to blood levels of patients taking these antibiotics (Larsson, 2007). When bacterial communities are exposed to such high levels of antibiotics, resistance levels increase dramatically within the bacterial population and are accompanied by genetic elements that can help these resistance genes move to other bacteria. Examining the resistance profiles of 93 pathogenic and non-pathogenic environmental bacterial strains from a WWTP receiving antibiotic manufacturing effluents in India showed that 86% of these strains were resistant to 20 or more antibiotics (Marathe, 2013). In addition, 95% of these strains harbored at least one mobile genetic element (an integron, or a piece of DNA that could transfer to a different bacteria and cause it to become resistant) (Marathe,

2013). Another study in China examined the resistance profiles of 341 environmental bacterial strains from a WWTP receiving discharge from an oxytetracycline production plant, river water downstream (RWD) to the WWTP and river water upstream (RWU) to the WWTP (Li , 2010). The percentage of oxytetracycline resistance strains from WWTP, RWD and RWU was 95%, 86% and 3%, respectively. In addition, 97% of the WWTP strains, 86% of the RWD strains, but two strains of RWU had mobile genetic elements, or integrons. Interestingly, the proportion of multi-drug resistant strains from both WWTP and RDW were also much higher when compared to RWU (96% vs. 28%). Recent studies indicate that a high percentage of multi-drug resistant strains, even in the presence of excess levels of a single antibiotic, is attributed to mobile genetic elements (especially plasmids and transposons, other types of DNA segments that can easily transfer antibiotic resistance genes to other bacteria) that contain multiple resistance genes (da Costa , 2013; Gonzalez-Plaza , 2017). Similar studies in India and China showed that antibiotic-resistant bacteria were abundant in rivers at the effluent sites of manufacturing units compared to upstream sites (Gonzalez-Plaza, 2017).

Antibiotic resistance patterns in pristine, low-level and high-level antibiotic contaminated environments

Although antibiotic-resistant bacteria are present in all three environments, the abundance of antibiotic resistance genes and mobile genetic elements were found to be much higher in environments with high-level antibiotic contamination (Bengtsson-Palme, 2014; Pal , 2016). One study compared abundance of resistance genes and mobile genetic elements in a recreational lake in Sweden not contaminated by sewage or industrial waste to a lake in India subjected to industrial pollution with fluoroquinolone antibiotics (Bengtsson-Palme, 2014). Antibiotic resistance genes were 7,000 times more abundant in the Indian lake compared the Swedish lake. Similarly, an abundance of mobile genetic elements were observed in the Indian lake samples when compared to the Swedish lake. In another study, bacterial populations in environments polluted with industrial antibiotic discharges carried the largest relative abundance and diversity of antibiotic resistance genes when compared to bacterial populations sampled from wastewater sludge, humans, or animals (Pal, 2016). Thus, available evidence strongly supports the notion that high-level antibiotic environmental contamination leads to an increased in the prevalence of antibiotic-resistant bacteria.

Risk to human health

We do not currently have a good understanding of how selective pressure in the environment causes the emergence antibiotic resistance and how this resistance is spread to humans (Bengtsson-Palme, 2017). There are two separate components in assessing the risk to human health– the risk of emergence of resistant organisms and the transmission of these resistant organisms to humans. The risk emerging antibiotic resistance in pristine environments is probably low; however our knowledge is limited. On the other hand, environments contaminated by industrial antibiotic discharge pose a considerable risk for resistance emergence especially where environmental bacteria are capable of colonizing the human body (Marathe, 2013; Li, 2010; Gonzalez-Plaza, 2017). Humans can be exposed to antibiotic-resistant bacteria through aquatic sources more often than from soil or air, but there is no direct evidence of transmission of antibiotic-resistant bacteria to humans from environmental exposure (Huijbers, 2015). Our knowledge on the impact of environmental exposures on human health is limited, despite reports of high levels of antibiotic-resistant bacteria and genes in aquatic sources impacted by industrial antibiotic discharges. Several studies reported that environments heavily polluted with antibiotics contain novel mobile genetic elements that are capable of inactivating last-resort antibiotics (Gonzalez-Plaza, 2017; Marathe, 2018; Berglund, 2017; Boulund, 2017; Flach , 2015) and one described the easy transfer of these mobile genetic elements to *Escherichia coli*, a bacterium that colonizes the human gut (Flach, 2015). A thorough understanding of how of antibiotic-resistant bacteria can spread in a variety of environmental settings and the impact on human health is urgently needed.

Little is known about the antibiotic resistomes, a bacterium's collection of resistance genes, of the vast majority of environmental bacteria, although the need is recognized for a greater understanding of environmental reservoirs of antibiotic resistance and potential impacts on clinically important bacteria and human health (Rosenblatt-Farrell, 2009; AAM, 2009). It is becoming increasingly clear that some environments harbor resistance genes irrespective of the human use of antibiotics, and the literature describes many cases of persistence of resistance genes in apparently antibiotic-free environments. These results show conclusively that antibiotic resistance is a natural phenomenon that predates the modern selective pressure of clinical antibiotic use.

C. Which measure are most important for limiting environmental contamination?

Emissions reduction from pharmaceutical manufacturing can be achieved through a combination of technical measures and incentivizing actions. Both approaches will be required to ensure a timely and

efficacious result, which is to limit or eliminate environmental contamination from antibiotic manufacturing.

Incentivising and Disincentivising Actions

Reductions in environmental contamination from pharmaceutical manufacturing can be driven by a range of legal, economic, and social incentives. These incentives can stem from numerous actors or stakeholders including regulatory authorities, governments, publics, media, international organisations (e.g., EU, WHO), investors, the pharmaceutical industry, academia, and insurance companies. Procurement practices must consider environmental issues within antibiotic sourcing across the whole of the supply chain—not doing so would be a significant disincentive for change. Heterogeneity in environmental stewardship across the industry has economic viability implications for antibiotic manufacturing and formulating. Antibiotic procurement needs to consider more than cost and quality; it must consider environmental stewardship across the whole of the product lifecycle.

There are opportunities for some members of the industry to be seen as leaders in environmental stewardship. Independent 'auditors', i.e., The AMR Alliance and the Access to Medicines Foundation, represent opportunities for an unbiased view of environemntal stewardship within the industry. The AMR Alliance has generated a framework for assessing environmental impact from manufacturing (https://www.amrindustryalliance.org/why-the-amr-industry-alliance/), while the Access to Medicines Foundation provides a series of benchmarks for the pharmaceutical industry, reporting on their transparency of systems and environmental stewardship, among other metrics (https://amrbenchmark.org/).

Incentivising transparency can be potentially critical when influencing change. Increased transparency about who is manufacturing the antibiotic, where it is formulated, and under what environmental conditions it is made can cause pressure on the supply chain. A suitable level of transparency will allow the consumer to follow the supply chain back to the manufacturer and make informed decisions about the sustainability of their manufacturing and supply chain. Providing this information allsows for an evidence-based procurement strategy by governments and health providers. Hence, transparency can be the agent of change. Where transparency is not maintained and systems are not independently auditable, procurement must respond by moving to providers that maintain these ideals. It is essential that 'Procurement' is transparent about the criteria that are required for sustained business, and it is

essential that these rules are maintained. Otherwise, the alternative is a 'race to the bottom' with regards to environmental stewardship, with cost being the primary incentive for procurement.

Currently, there are no antibiotic discharge limits. Where countries might impose a discharge limit, it creates a disincentive for manufacturing because it can cost more to achieve these discharge limits than using an equivalent manufacturing facility in another country. This highlights the importance of globally implementing an agreed upon discharge limit. Having a transparent and independently auditable supply chain is the only mechanism to enforce such a discharge limit because each country's environmental regulatory processes will vary.   TThe best way to achieve this is through consumer-driven incentives, i.e., those within the industry that do not comply are not eligible for government procurement. tablishing and maintaining a consumer-driven incentivisation system can be relatively easy to maintain with "Green" labelling initiatives—a method that quickly demonstrates compliance with transparency and stewardship benchmarks.

Stewardship Actions

Reducing patient and animal consumption through effective stewardship (e.g. appropriate diagnosis and prescription) will likely reduce the environmental selective pressures for AMR both through reduced excretion from patients and less production. If 20-30% of antibiotics are used inappropriately (Davies, 2018; O'Neill & The Review on Antimicrobial Resistance, 2016), this is a good option to reduce overall environmental exposure and selection in a clinical context at the same time. However, this is only a partial solution for reducing environmental loads, as the quantity of antibiotics that are deemed necessary is still very high and will continue to increase with an aging global population.

Technical Actions

Techniques designed to remove pharmaceutical residues from waste streams already exist and are applied in some settings (for example, standard biological or chemical treatment, activated carbon, oxidative agents, reverse osmosis, evaporation and incineration). Depending on the regulatory emission targets that need to be met, further innovation in industrial and domestic effluent treatment technologies may be needed. Consideration will need to be given to the risk of using biological treatment for pharmaceutical manufacturing waste. This approach will enrich for antibiotic- resistant bacteria, which would enter the environment if no further treatment of the waste stream is employed.

Emission targets for antibiotics and antibiotic-resistant bacteria will need to be established concurrently to avoid resistance selection and dissemination in the environment.

A regulatory emissions target for antibiotic resistance genes will necessitate the use of non-biological treatment methods or subsequent destruction of the genes after biological treatment. This endpoint could be cost-prohibitive making biological wastewater treatment impractical. Fermentation wastes will have particular issues e.g. high biochemical oxygen demand, or BOD, wastes and mycelia mats. The antibiotic residues found within mycelia mats might preclude their disposal through existing routes, such as re-use on animal farms, because of the risk for selection of resistance in the animals and their environment. There is a need for the industry to determine what is the 'Best Available Technology' (BOT), e.g., 'zero liquid discharge' policy.

D. What is the economic impact of implementing known measures to prevent environmental contamination?

It is well established that antibiotic resistance is a natural phenomenon in the environment. The anthropogenic, or human-caused, impactof pollution on this natural reservoir of resistance has greatly increased the likelihood that novel resistance genes could spread from the environtment to clinically-relevant microorganisms.

At the United Nations General Assembly (UNGA) roadmap group, thirteen signatory companies involved in the production and marketing of antibiotics responded to the WHO call for a more transparent, responsible, and accountable pharmaceuticalsupply chain to combat antimicrobial resistance (see Annex 1). The AMR Industry Alliance (AIA) is the life-sciences response to the UNGA call for industry action to action, which ensures that signatories collectively deliver on the specific commitments made in the UNGA Declaration (January 2016) and the Roadmap (September 2016) and measures industry's progress in the fight against antimicrobial resistance. AIA is made up of more than 100 biotech, diagnostics, generics, and research-based biopharmaceutical companies and trade associations. In the AIA progress report (18 Jan 2018), this workgroup has reported on voluntary measures taken by the AIA members to combat AMR. AIA is working towards establishing science-driven, risk-based targets for discharge concentrations for antibiotics and good practice methods to reduce the environmental impact of manufacturing discharges. The AIA aims to achieve these targets by 2020. At present, discharge limits are based on traditional

predicted no-effect concentration (PNEC) values. Thisa measure has no relevance to the selection of resistance genes andis not fit for the purpose of limiting the spread of AMR.

Handling of waste with antimicrobial activity

The composition of manufacturing waste will be a complex mixture of different active pharmaceutical ingredients (APIs) depending on the facilitywhich might produce a range of different drugs. The range of APIs at any one facility would be mixed with impurities, solvents, buffers, biocides, catalysts, metals, and potentially microorganisms.

Several methods have been described in the literature for handling hazardous pharmaceutical manufacturing waste, with the most complete method being incineration. Incineration can be effective in eliminating all antimicrobial activity. Proper treatment of off gas is required to limit environmental impact. This is the most effective but likely the most energy-intensive method.

More innovative methods for the reduction and potential elimination of the antimicrobial properties of pharmaceutical wastewater include:

- Microbiological treatment – including the aerobic or anaerobic decomposition of organic components in the waste stream. Where applied, this can be very effective, but potentially incomplete because there are lower-limit thresholds which could limit its success. It also has the disadvantage of being susceptible to highly toxic components of the waste stream.

- Enzymatic treatment – includes the use of specific enzymes that degrade chemicals in the waste stream. This method does not require live microorganisms, so it is less affected by toxicity issues. It also has a low risk of contaminating the downstream environment because the enzymes will naturally degrade, unlike with microbiological treatments.

- Chemical treatment – this method chemically decompose the organic components within a waste stream using using an acid, base, Fenton oxidation (using free radicals to oxidize a compound), ozone, or chlorine. The waste stream would likely require neutralization and secondary treatment to address the dissolved organic load.

- Absorption – this method would allow for the removal of organic compounds from the waste stream by partitioning them from the aquatic phase to a solid, such as activated carbon. This method can be effective for a wide range of chemicals but can be expensive.

- Photocatalysis – this method uses a specialized piece of equipment called a photoreactor to generate light and free radicals which treats the waste.
- UV light – this method replicates the effective UV light emitted by the sun, which effectively degrades many environmental pollutants. The sun's activity can be replicated in a wastewater treatment facility degrade chemicals kill microorganisms.
- Electrochemical degradation – this is an effective method that oxidizes organic compounds in wastewater. This method is followed by secondary treatments like UV and chemical treatment.

The selection of the most economical route to treating API manufacturing wastewater with antimicrobial activity is dependent on:

- Composition of the waste stream
  - o Is the stream water-based or solvent-based
  - o How easily can the waste be biodegraded
  - o Are there metals present in the waste stream
  - o What is the concentration of APIs, impurities, etc.
- To what level the concentration of antibiotics needs to be reduced (what is the desired target)
- Volume of the waste stream

Economic Evaluation of Treatment Methods

The acceptable level of the antibiotic after the treatment determines to a large extend the cost of treatment. Discussion is on-going about acceptable levels of antibiotics in the receiving environment. Companies that responsibly produce antibiotics have set their own limits, mainly based on eco-tox data (PNEC-E) or on bio-assays. However, these limits do not predict acceptable levels for minimizing the risk of developing antibiotic resistance. One of the actions of the Workgroup Manufacturing of the AMR Industry Alliance is to set science-driven, risk based targets (see Annex 1).

In general, biological treatment is the most economic method for treating waste; however, during this treatment, a population of microorganisms may develop that have the ability to degrade antimicrobial compounds and thus have antimicrobial resistance genes (ARG's). The level of ARG's in effluent and the increase of resistant pathogenic microorganisms is not understood. Proper handling of surplus sludge and effluent treatment from the waste water treatment plant may be required. It is also important to recognize that the microorganisms can be lost if the waste stream becomes too toxic. In the event the

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waste stream contains compounds that could kill the microorganisms, these must be removed using another treatment method (e.g., advanced oxidation) prior to microbiological treatment. It is also likely that the effluent will contain compounds with antimicrobial activity, necessitating additional treatment (e.g., carbon treatment).

The issue of ARG's is not limited to treatment of manufacturing site wastewater. Treatment of municipal wastewater results in effluent released to the environment with a large number of ARG's. In a report (published in 2017) of the National Institute of Health in the Netherlands (RIVM) an analysis of effluents of all WWTP's in the Netherlands resulted in a release of > 2. 10^{20} copies of SUL-1 genes every year into the environment. Levels of other ARG's (in literature > 100 types have been described) have not been published by the institute.

Incineration is the best method for waste streams with high amounts of organic solvents or other organic compounds. Waste streams with high levels of inorganic material (mainly salts) are usually treated with a multi-step evaporation system. The antimicrobial compounds in the waste stream may be eliminated during this process. Otherwisethe waste stream needs to be treated prior to the process. The water coming from the unit needs to be treated (microbiological treatment) and disposal of the solids need to be done in line with the local regulations, normally dispensing to a landfill.

Cost of treatment

Unfortunately, it is difficult to get public data on the cost of antibiotic manufacturing waste treatment. The cost depends on:

- Type of compounds to be eliminated
  - The accepted level of antibiotic in the environment
- Type of technology required for treatment
- Volume of the product and waste stream
- Manufacturing location

In many cases, the operational cost can be reduced by investing in advanced equipment for treatment. A ballpark figure is that 15% of the cost of the API or intermediates is the total cost (cost of depreciation of the investment and operational cost) to ensure that the level of the antibiotic does not exceed the PNEC value of that antibiotic in the receiving environment.

As mentioned before, PNEC-E value is based on eco-tox data and not necessarily the correct figure to prevent the development of drug-resistant microorganisms. In literature, values of PNEC, which relates to resistance selection,of several antibiotics have been published and are in a range of 10 – 10.000 ng/l. Also some data are available on the determination of minimum selective concentration (MSC). The discussion on what is a science-based limit of antibiotic in the environment is ongoing and is one of the targets of the manufacturing working group of the AIA (ANNEX 1). Further study is needed to establish maximum limits on antibiotics and ARG's in the environment to minimize the risk of development of resistant pathogenic microorganisms, and due to the multiple factors involved, determining a more precise estimate of cost above a range is difficult.   One of the targets of the Workgroup Manufacturing of AMR Industry Alliance is to work with independent technical experts to establish science-driven, risk-based targets for discharge concentrations for antibiotics and good practice methods to reduce environmental impact of manufacturing discharges, by 2020, which will help inform assessments of economic impact

E.   Is a standard method for measuring environmental contamiation established?

Lack of standardized methods and regulations for monitoring antibiotic manufacturing wastes

The pharmaceutical industry generates wastewater discharges of varying characteristics and contaminant concentrations depending on the nature of the production process. The main chemicals present in these effluents are solvents, detergents, disinfectants, and pharmaceutical products, all of which are potentially ecotoxic. However, while there are standard methods for monitoring volatile organic compounds (e.g. EPA method 1671 using gas chromatography with flame ionization detection) and other water-soluble organic compounds such as formaldehyde, isobutyraldehyde, and furfural (e.g. EPA method 1667, using high performance liquid chromatography), there are no standard methods for the analysis of residues of (APIs and their transformation products that may be formed during wastewater treatment. The lack of standard method for API analysis in manufacturing wastes is an important gap in investigating the sources and mechanisms of antibiotic resistance in the environment because the concentrations of antibiotics in manufacturing wastes have been reported to be significantly higher (in mg per liter concentrations) than the concentrations observed in the effluents of municipal wastewater treatment plants (typically in sub µg per liter concentrations) (Larson, 2014).

While antibiotics released into the environment are considered to be important drivers of AMR development and proliferation, antibiotic manufacturers are not required to report concentrations of APIs released in their wastewater discharges. Due to the polar nature and low volatility of antibiotics, analysis of these compounds in environmental and biological samples is commonly performed using liquid chromatography (LC) coupled with mass spectrometry (MS) detection, which provides high degree of selectivity and sensitivity. However, the accuracy of LC-MS analysis can suffer significantly from either signal suppression or signal enhancement due to the presence of co-extracted components of the sample matrix that interfere in the chromatographic separation and ionization process in LC-MS. The extent of matrix effects on the signal intensities of target molecules vary greatly, and depends both on the nature of the molecules and the composition of the matrix inferences (e.g. humic acids, proteins, phospholipids). The most frequently used method for antibiotic detection involves an LC with a triple quadrupole MS operated under selected reaction monitoring mode, resulting in a selective tandem mass spectrometry analysis (LC/MS/MS) (Diaz-Cruz, 2006; Seifrtová, 2009). More recently, advances in instrumentation has achieved even faster and more selective analysis of multiple classes of antibiotics in aqueous samples using ultra-high pressure LC coupled with hybrid quadrupole-linear ion trap MS detection systems (Gros, 2013). The LC-MS methods are highly sensitive, with method quantification limits (MQL) reaching sub-ppt levels (1-100 ng per Liter), depending on the type of antibiotics and the complexity of sample matrices. These methods allow for multi-residue analysis, e.g. 100 compounds or more can be analyzed within a single short (e.g. 30 min) analytical run, potentially including all key antibiotics, their metabolites, transformation products, and other co-selecting agents such as biocides. While less common, the use of gas chromatography coupled with mass spectrometry (GC-MS) has also been reported (Hao, 2007), but the application of GC-MS is limited to antibiotics that can be derivatized to volatile forms. Most analytical laboratories within pharmaceutical and water sectors own or have access to accredited labs with LC-MS or GC-MS capability.

Published analytical methods for antibiotic analysis usually provide robust validation data to assure reproducibility and accuracy of results. However, these methods are not standardized and vary from one laboratory to another. Most data on the occurrence of antibiotics in the aquatic environments are derived from surface waters receiving discharges from municipal and hospital wastes or agricultural run-offs. In addition, most data result from localized research projects that are usually supported by national funding agencies or research foundations. Because there are no government regulations for antibiotic manufacturers to provide information on the residual concentrations of antibiotics, their metabolites and

degradation products data on the occurrence of antibiotics in manufacturing wastes at the national and global scale are difficult, if not impossible, to obtain. Therefore, new funding strategies are needed to support data collection on the antibiotic concentrations in manufacturing wastes, using standardized methods that are robust, comprehensive, and fit for purpose.

Challenges and Limitations of Current Analytical Methods for Antibiotics

Analysis of antibiotics in environmental samples using LC-MS is subject to a variety of interferences from matrix components (e.g. high concentration of salts, dissolved organic compounds, proteins and fatty acids) that could result in false-positive and false-negative detections. In fact, quantification of antibiotics in manufacturing wastes may be prone to errors due to high concentrations of precursors of active pharmaceutical ingredients, fermentation by-products, or side-products of chemical synthesis. Additional challenges – not confined to the analysis of manufacturing waste, but also common to any environmental analysis for antibiotics–include poor extraction recoveries, ionization suppression in LC-MS, and unpredictable matrix effects (Aga, 2016). Therefore, it is critical to use isotopically-labeled analogues of antibiotics as surrogates during analysis of manufacturing wastewater to compensate for the variability in the extraction recoveries and matrix effects. Unfortunately, not all antibiotics have commercially available labeled analogues, in which case, an internal standard that is structurally related to the target antibiotics should be used as surrogate to account for losses during sample preparation, and quantification. In addition, performance criteria should be established for the LC-MS methods, such as setting acceptable tolerance in the deviations from expected ratios of the qualifier and quantifier ions, or acceptable retention time shifts in the chromatograms. Finally, the effect of sample storage and sample preparation on the antibiotic stability should be evaluated because it is not known whether the storage temperature and length, or the chemical additives (e.g. acidification of samples) used prior for filtration or sample extraction will affect the integrity of the analytes.

The concentrations of antibiotics in surface waters receiving discharges from municipal wastewater treatment plant effluents are typically found at low concentrations (at sub µg/L levels), and therefore require extensive sample preparation and concentration. Solid phase extraction (SPE) is the method of choice for the extraction of antibiotics from liquid matrices such as river water and wastewater (Diaz-Cruz, 2006; Moreno-Bondi, 2009). Generally, in all types of waters analyzed, SPE recoveries achieved for target antibiotics ranged from 50 to over 100%; low recoveries may result from highly polar antibiotics with low sorption to the SPE cartridge. However, because the concentrations of antibiotics in manufacturing

wastes are expected to be high (at mg/L levels), it may be possible to perform a "dilute-and-shoot" analysis, where no sample clean-up or concentration is performed, eliminating the potential to lose some analytes during SPE. In a "dilute-and-shoot" approach, only a 10-fold or a 100-fold dilution of sample is required prior to injection, making it ideal for high-throughput analysis of antibiotics in manufacturing wastes. However, before a "dilute-and-shoot" method can be implemented, it is critical to establish the target quantification levels for the antibiotics and other analytes of interest in the manufacturing waste to determine if the MQL of the method is sufficient to detect the target concentrations. However, because there are no regulations on what are the allowable maximum contaminant levels of API residues in the discharged manufacturing wastes, it is not currently possible to recommend the use of "dilute-and-shoot" method as an acceptable cost-effective alternative to the time-consuming SPE procedures used in traditional methods.

Because some fraction of antibiotics can sorb in the sediments of receiving waters, or in the biosolids of fermentation broths from the manufacturing wastes, it is also important to determine the concentrations of antibiotics in solid samples. The extraction of antibiotics from solids (suspended particulate matter, sediments and biota) can be achieved using different techniques, ranging from simple sonication of the solid samples with organic solvents, to using accelerated solvent extraction and microwave assisted extraction for more aggressive extraction conditions (Petrovic, 2005; Diaz-Cruz, 2006; Speltini, 2011). Extraction of antibiotics from solid matrices is troublesome and therefore many large monitoring campaigns focus only on liquid phase, which does not help with furthering our knowledge about how antibiotics cycle in the environment. Future monitoring strategies should therefore consider solid matrices, including suspended particulate matter, sediments, and biota.

The biggest limitation of the current analytical approaches is that they are limited to analyzing a few known target analytes. For instance, only the active pharmaceutical ingredients or the parent antibiotics are commonly included in the analytical method, and thus, potential transformation products formed during treatment or upon disposal to the environment are not considered. However, some classes of antibiotics are unstable in the environment and form transformation products that may still be biologically active. For instance, tetracyclines are known to epimerize or hydrolyze (Aga, 2005), or form photodegradation products that retain the conjugated tetracycline rings (Eichhorn, 2004) suggesting that these transformation products are still biologically active. In addition, antibiotics in the β-lactam family (e.g. cephalosporins and penicillins) are generally unstable because of the susceptibility of the β-lactam

bond to hydrolysis. Therefore, it is important to include epimers of tetracyclines, and hydrolysis by-products of β-lactams in monitoring API residues in manufacturing wastes. Few papers report that some metabolites are present in the environment at higher levels than their parent compounds (Diaz-Cruz, 2006).

Recently, an increasing number of publications have reported the use of high-resolution mass spectrometry (HRMS) for environmental monitoring in an attempt to move away from target-driven analysis. Liquid chromatography coupled to high-resolution MS, such as quadrupole time-of-flight (QToF) MS and Orbitrap™ MS allow for target analysis to be undertaken alongside with non-target screening, and more importantly the possibility for retrospective analysis. This approach could revolutionize the way we approach environmental issues through storage of long-term datasets subject to retrospective analysis when and where needed in line with furthering the research questions posed. The ability of QToF MS instruments to acquire full mass range spectra without sacrificing speed or sensitivity makes these types of instruments an excellent choice for qualitative and quantitative analyses across a wide range of antibiotics classes in the presence of complex matrices. However, while the high resolving power of QToF MS provide high degree of selectivity via exact mass measurements, this MS format has generally lower sensitivity when compared to triple quadrupole MS when running under selected reaction monitoring mode. On the other hand, the Orbitrap™ MS overcomes many of the limitations of the other LC-MS instruments because it can use the synchronous full-scan MS and MS/MS acquiring capabilities which are advantageous on both confirmation and quantification. While the QToF MS can also perform full-scan MS and MS/MS experiments, the Orbitrap™ MS has a much faster data acquisition rate that can provide low detection limits and higher sensitivities, allowing detection of low signal intensity ions on antibiotics and their transformation products. However, the cost of Orbitrap™ MS is about twice as much as the other MS platforms and has hindered this instrument from being commonplace in many environmental laboratories. Therefore, HRMS technologies are still considered as research tools with very limited applications in environmental regulatory settings.

Need for Complementary Bioanalytical and Molecular Assays to Assess Impacts of Manufacturing Wastes

Environmental issues require comprehensive evaluation of the environment in question via combined bioanalytical approaches encompassing both exposure and hazard analysis. In the context of AMR, this would require combining mass spectrometry (targeted vs screening/retrospective) focusing on chemical

targets with bioanalytical approaches focusing on either known resistance genes (via PCR analysis) or trying to undertake whole genome screening. In addition, ecotoxicity tests using whole organisms (fish assays), bacteria, or cell toxicity assays should be implemented as part of the standard test.

Monitoring antibiotic resistance genes in environmental matrices has recently been recommended, with the increasing recognition that these genes can, in and of themselves, represent 'emerging contaminants' (Pruden, 2006). Molecular analyses of environmental samples to identify the presence and diversity of resistance genes could potentially become very useful in identifying hotspots of AMR locally and on a global scale (Luby, 2016). Due to its ability to capture the signature of the entire metagenome of environmental samples, genomic data holds the most promise for revolutionizing environmental AMR studies as it can provide a comprehensive understanding of resistance genes and their correlation to associated bacteria. Though genomic research tools are more accessible to researchers in developed countries, the falling cost of metagenomic sequencing is increasing the utility of this tool in unraveling the complexities of antibiotic resistance.

As AMR is a global challenge, global networks for monitoring of AMR determinants need to be established to fully understand the dynamics of AMR in the environmental context. One vital aspect of global data collection is robust interlaboratory comparison and standardization of sampling, sample preparation and analytical approaches. This needs to be preceded by comprehensive evaluation of key AMR determinants and selection of AMR markers for localized and global monitoring.

F.   What information is needed to establish a standard for acceptable waste discharge from a manufacturing facility?

Response not received from subject matter experts.

III. LITERATURE REVIEW

A. To what extent is the environment currently being contaminated with antibiotics from manufacturing waste?

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- No references at this time

Antimicrobials as Pesticides

Prepared by Professor Stephane Bayen (McGill University), Dr Hubert Dirven (Norwegian Institute of Public Health), Dr Brendan Jackson (Centers for Disease Control and prevention), Professor Jeff LeJeune (Ohio State), Professor Randall Singer (University of Minnesota), Dr Virginia Stockwell (USDA), Professor James Tiedje (Michigan State)

I.   BACKGROUND STATEMENT

Antimicrobials, including antibiotics and antifungals, are widely used as pesticides for crop management. In some cases, these antimicrobials are the same, or closely related to, antibiotics used in human medicine (e.g. tetracyclines, aminoglycosides, and triazoles). The use of antimicrobials as pesticides has the potential to select for resistant microorganisms present in the environment. This is of particular concern if the microorganism can cause human infection or confers transferable resistance mechanisms to antimicrobials commonly used to treat human infections. For example, use of streptomycin, has the potential to select for resistance, like than encoded by 16S-methylases, which can confer resistance to all aminoglycosides used in human medicine including a new aminoglycoside in the pipeline, plazomicin. Antibacterial use as a pesticide is not known to select for resistance affecting human health, but vigilance is needed, especially in cases where antibacterial use increases or when the environment exposed to the pesticide is contaminated with multi-drug resistant bacteria.

In the last decade, infections with *Aspergillus fumigatus*, a fungus common in the environment, resistant to all triazole antifungals, were detected first in Europe and now across the world. This fungus, which infects humans through inhalation, causes severe and often fatal invasive mold infections in the growing proportion of the world's population that is immunocompromised. Triazole fungicides are used widely in plant agriculture, representing the largest class of fungicides in some countries (https://water.usgs.gov/nawqa/pnsp/usage/maps/ and http://www.fao.org/faostat/en/#data/RP). In human medicine, triazole antifungal medications structurally related to fungicides are used to treat not just superficial skin infections but also many life-threatening fungal diseases. Triazole antifungals have become the mainstay of therapy for these infections; however, these medications are ineffective against resistant strains, resulting in a near doubling of the mortality rate to about 90% (Verweij, 2016). Several lines of evidence suggest that agricultural and other environmental triazole use has caused the most common type of pan-triazole-resistant *A. fumigatus* infections known as TR34/L98H (http://ecdc.europa.eu/en/publications-data/risk-assessment-impact-environmental-usage-triazoles-

development-and-spread), (Snelders, 2012; Chowdhary, 2018). Notably, the majority of patients with resistant infections have lacked previous exposure to medical triazole antifungals (van der Linden, 2011), suggesting that they became infected with a strain already carrying these mutations.

When evaluating the risk of antimicrobial use as a pesticide on human health, it is important to assess how likely an antimicrobial is to select for resistance to the drug itself, resistance to related drugs (i.e., cross-resistance), or resistance to unrelated drugs because of genetic linkages between resistance determinants (i.e., co-selection of resistance). It is also important to understand the extent to which antimicrobials used as pesticides can contaminate the environment beyond the field borders and how long the antimicrobial is active in the environment. Efforts to mitigate the risk of using antimicrobials as pesticides will require knowledge of the extent to which drugs are used, application strategies with proven effectiveness in limiting risks to human health, and strategies that can be used to reduce or eliminate the need to use antimicrobials on crops.

II.   SCIENTIFIC ISSUES

A.   What is the current landscape of antimicrobial use as pesticides; which drugs and how much?

This section first will describe the antimicrobial chemicals, including antifungals, applied to crops for management of plant diseases that are the same or closely related to antibiotics used to treat human infections (Table 1). Some of these antibiotics are also used in animal agriculture. Antibiotics used on plants that are not used clinically or on animals will not be addressed here. Copper formulations, which are the most commonly deployed antibacterial pesticide for diseases of plants, are not used in humans. However, copper and other general biocides may be involved in co-selection of antimicrobial resistance determinants. In this section, the term antibiotic is used synonomously with antibacterial and distinct from antifungal, although some definitions of the term antibiotic encompass antifungals.

Why are antimicrobials used on crop plants?

Antibacterial chemicals

Following the discovery of antibiotics, several compounds (e.g. penicillin, streptomycin, aureomycin, chloramphenicol, and oxytetracycline) were evaluated for their ability to control of bacterial diseases of plants (McManus et al. 2002). Of the antibiotics tested, streptomycin provided excellent control of several bacterial diseases of plants when applied at low doses (100 ppm). Streptomycin was non-toxic

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to plants and did not cause undesirable markings on fruit and, in the United States, it was the first antibiotic registered for plant protection in 1958. Bacterial diseases of plants are difficult to control and can be extremely damaging. Some bacterial plant pathogens are seed- or tuber-transmitted, whereas many others are present in the environment and overwinter in infected tissues. Generally, antibiotics are used to control bacterial diseases of high-value crops, primarily tree fruits. One requirement for a bacterial plant pathogen to infect a plant is a fresh wound on a plant surface or a natural opening, such as stomata or secretion pores. These wounds or natural openings are infection sites that permit the bacterium to gain access into the internal tissues of the plant. The wounds allowing ingress of bacterial pathogens can occur due to weather events (e.g. freeze damage, driving rain, hailstorms, and wind), insect activity, and horticultural practices, such as driving machines through cropped areas or pruning trees. For many bacterial plant diseases, another important step in pathogenesis is the epiphytic growth phase, during which the pathogen multiples on the plant surface to large population sizes (~106 colony forming units) prior to infection of tissues. During this epiphytic growth phase, the growth rate of the pathogen is influenced by environmental conditions. If environmental conditions are not conducive for rapid epiphytic growth, the probability of successful infection is reduced.

Bacterial plant pathogens are exposed on plant surfaces and vulnerable to disease control methods during the pre-infection epiphytic growth phase. Antibiotics generally are applied as a prophylactic treatment to disrupt the epiphytic growth phase of plant pathogenic bacteria and prevent subsequent infection. The use of antibiotics after disease symptoms are visible is discouraged because 1) antibiotics are not curative when sprayed on infected plants and 2) potential for selection of antibiotic-resistant plant pathogens increases as the population size of the bacterial pathogen in host tissues increases.

Antifungal chemicals

Fungi compose the largest group of plant pathogens, and fungicides are used widely in plant agriculture to prevent and treat fungal diseases. Among fungicides, triazoles are the largest and most widely used class, having broad-spectrum antifungal activity (http://www.apsnet.org/publications/apsnetfeatures/Pages/Fungicides.aspx). They are used on a diverse range of plants, previously largely in high-value crops, such as orchard trees and grapes, and increasingly on commodity crops, such as wheat, corn, and soybeans. For example, in the United States, the low estimate of triazole use on orchards and grapes (134 metric tons) in 1995 was more than double use on wheat and corn (no use was reported on soybeans), whereas in 2015, the preliminary low

estimates for use on wheat (1,068 metric tons), corn (331), and soybeans (206) exceeded use on orchards and grapes (202) (https://water.usgs.gov/nawqa/pnsp/usage/maps/).

An example of regulation of antibiotics and other pesticides used on crops

The US Environmental Protection Agency (EPA) is the federal regulatory agency for materials applied to plants in the United States. Many countries have similar agencies to regulate which materials may be used for plant production. In the US, each active ingredient is registered for use on a specific crop or crop group. For example, the crop group 'Pome fruit' includes apple, crabapple, mayhaw, Asian pear, quince, Chinese quince, Japanese quince, and European pear and a material registered for the Pome fruit group may be used on any of these plants. Whereas, other materials may be registered for just a single member of the Pome fruit group, like European pear, and would be restricted for use only on pear trees. Individual states may introduce additional restrictions on pesticide use that would only apply to their state. The EPA evaluates the environmental impact and possible detrimental effects of the active ingredient and formulation materials at a proposed dose on humans, animals, insects, and aquatic systems prior to granting a registration for a material for crop health. Additionally, the EPA establishes the Maximum Residue Level (MRL) of the pesticide permitted on the harvested crop. The EPA-approved materials, instructions for use, and limitations are stated on product labels. The restrictions listed on the product labels for materials used on crops are legally binding.

Publically available sources of pesticide use data in the United States

Two agencies within the US government have collected data on all materials applied to plants or agricultural soils since the 1990's; the USDA National Agricultural Statistics Service (NASS) and the US Geological Services National Water-Quality Assessment Project. The National Water-Quality Assessment Project of the US Geological Services (USGS) tracks pesticides in surface and ground waters in the USA to estimate the potential for pesticides to affect aquatic ecosystems and drinking water sources. A component of this project involves generating estimates of annual agricultural use of pesticides from confidential pesticide use reports and the harvested crop acreage surveys of specific farms located in US Department of Agriculture (USDA) Crop Reporting Districts. The proprietary farm-specific data are used to project pesticide use in larger regions based on acreage of crops in a region, as reported by the USDA Census of Agriculture (Baker and Stone, 2015). Annual high and low estimates of pesticide use by the USGS are provided at https://water.usgs.gov/nawqa/pnsp/usage/maps/county-level/.

The second source of pesticide use data is the USDA National Agricultural Statistics Service (NASS). The NASS Chemical Use data provides information on on-farm chemical use and pest management practices.

The chemical use data are collected directly from farmers and include information on actual on-farm use of materials, such as the annual amount of the active ingredient of a pesticide is used, the number of applications of a material, and the percentage of acreage treated. Data for materials applied to crops is available in the on-line QuickStats database at https://quickstats.nass.usda.gov/.

[Data sources of pesticide use outside of the United States needs to be investigated]

Types of antimicrobials used

Antibacterial Chemicals used in crop plants

Streptomycin (CAS 57-92-1). Streptomycin an aminoglycoside in resistance group 25. Streptomycin has been used in the US since the 1950s for management of bacterial diseases of plants. Streptomycin is registered for control of several diseases on plants, such as the prevention of rots and other diseases on seed potatoes, tomato and tobacco transplants, and stems of cut flowers in the US, except for the state of California. For these diseases, streptomycin is applied to seed pieces or transplants in greenhouses or other structures, prior to planting in the field environment. After planting outdoors, the application of streptomycin is limited or not allowed. The registered uses of streptomycin on crops in the U.S. are summarized in Table 2.

More than 90% of the quantity of streptomycin used for crop protection is applied to pear and apple orchards for the prevention of fire blight caused by *Erwinia amylovora* (Stockwell & Duffy 2012). Streptomycin also is registered for fire blight management in Canada, Israel, Mexico, and New Zealand. Streptomycin was used for fire blight control in Austria, Germany, and Switzerland on a strictly-regulated, emergency use basis for fire blight prevention until ~2015, after which this material is no longer approved in Switzerland and the EU (Duffy and Stockwell, 2012; Gusberti *et al.*, 2015).

Fire blight is the most destructive bacterial disease of pear and apple. Trees are most vulnerable to infection by *E. amylovora* during bloom in the spring months. The bacterial pathogen survives the winter months in cankers, which are infections on the trunk and stems of trees. In the spring, pathogen cells exuded from cankers in an ooze matrix and spread to open flowers by insects, wind and rain. The pathogen colonizes the nutrient-rich floral stigmas and rapidly develops population sizes exceeding 10^6 colony forming units per flower under favorable weather conditions (Thomson, 2000). Free moisture (rain or heavy dew) aids movement of the pathogen to the nectary tissue of the flower, the site of infection. The nectarthodes (nectar secreting pores) are the entryway for *E. amylovora* to invade the

plant tissues. Inside the intercellular spaces of the flower, the pathogen produces effector proteins that kill plant tissues, while migrating down the floral stem into the branches. Soon, flower clusters are killed and the symptoms of fire blight are visible. At this stage of fire blight, diseased and surrounding healthy tissues should be removed to reduce the internal spread of the pathogen. Secondary phases of the disease include infection of young shoots or fruits. Young trees in orchards or nurseries are especially vulnerable to fire blight. The spread of fire blight from infected branches from floral or shoot infections to the trunk can be lethal. Regional losses to growers during widespread outbreaks of fire blight are estimated in the range of $40 million to $70 million million (Loper *et al.*, 1989; McManus, *et al.*, 2002). It was estimated that growers across the US spend at least $100 million annually to fight this disease (Norelli *et al.*,2003).

The discovery that streptomycin was effective against fire blight provided growers a method to control the disease. The epidemiology of the pathogen and the disease were not well understood in the 1960's and growers tended to spray streptomycin frequently during the growing season. Failures of streptomycin to control fire blight were reported within 20 years after streptomycin was first used in pear and apple orchards for fire blight control (Schroth *et al.*, 1978). Streptomycin resistance in *E. amylovora* has been reported subsequently in many regions of the US, Canada, Israel, Mexico, and New Zealand (Jones and Schnabel 2000; Loper *et al.*, 1991; McManus *et al.* 2002; Smits *et al.* 2014). Frequently, streptomycin resistance in *E. amylovora* is due to a spontaneous mutation in *rpsL*, which leads to a substitution of lysine to arginine at codon 43 [K43R] (Chiou and Jones 1995). In Michigan, isolates of *E. amylovora* also gained resistance to streptomycin via an acquired tandem gene pair *strA-strB*, which encodes for an aminoglycoside phosphatase that inactivates the antibiotic (Chiou and Jones 1995a; McManus *et al.* 2002).

Despite the potential for resistance to streptomycin, the antibiotic is still used in pear and apple orchards and remains one of the best chemical controls for fire blight against sensitive isolates of the pathogen. To mitigate resistance, streptomycin often is applied in combination with or rotated with oxytetracycline in U.S. tree fruit orchards. In Latin America countries, streptomycin is sold as a single active ingredient, combined with oxytetracycline, or combined with oxytetracycline and copper (Table 3).

Estimates on streptomycin use for crop protection on commercial farms in the US were obtained from the US pesticide use databases. The USGS estimated that in 2015 between 18,000 to 19,800 kg a.i. streptomycin was applied to crops. Figure 1 provides a summary of streptomycin use from 1991 to 2015

in the US from the NASS QuickStats database. Generally, the quantities and usage patterns of streptomycin were similar over the 24-year period. Streptomycin usage in 2015 is summarized in Table 3. In 2015, 92% of the quantity of streptomycin used on tree fruits was applied to apple. Total amounts of streptomycin sprayed on crops provides general information about pesticide use. Nonetheless, it is important to consider the average number of applications during a growing season and the percent of the orchard acres that were treated. In Table 3, streptomycin was applied twice on average to 26% of the total apple acreage in 2015. Pears were treated an average of three times during the season on 16% of the acreage in 2015 (Table 3). Even though apple trees were sprayed less frequently with streptomycin that pear trees, the much larger acreage of apple orchards (136,358 HA) accounts for the greater total quantity of streptomycin that was used on apple compared to pear (20,823 HA)(Table 3).

Overall, the total amount of streptomycin applied to pear and apple orchards is a fraction of the total amount permitted on product labels (Table 2). On the product labels, streptomycin may be applied 10 to 15 times during a season on 100% of the acreage. The low actual use of streptomycin by growers is, in part, due to the use of fire blight decision aids and disease risk models. The models estimate disease risk and when growers should intervene with antibiotic treatment using the following parameters: the recent history of fire blight in their orchard or surrounding orchards, the occurrence of environmental conditions are conducive for rapid growth of the fire blight pathogen on floral tissues, and the presence of open flowers on trees (Billing, 2000; Lightner and Steiner, 1992; Smith 1993; Thomson, 2000). The decision aids help growers optimize the timing of streptomycin sprays to periods when they will be most effective and also reduces excessive use of streptomycin and selection pressure for resistance.

Recently, the US EPA granted emergency use registrations for streptomycin on citrus in Florida and in limited, specific regions of California for management of the disease citrus greening. The emergency use registrations may be granted in response to applications from individual states for specific crops. The emergency use registrations are time-limited and the quantities and methods for use of streptomycin are regulated and specified on special use labels. Usage data on streptomycin on citrus under these restricted emergency uses are not publically available at this time.

In addition to formulated streptomycin products used on commercial farms by certified pesticide applicators, agricultural streptomycin is available for residential use in products marketed for home gardens (for example, Fire blight Spray, Fertilome, Bonham, TX). These minor uses of streptomycin in non-commercial agricultural settings would not be captured in the USGS or USDA NASS databases. It is unknown how much streptomycin is used in home garden settings by homeowners.

Oxytetracycline (CAS 79-57-2). Oxytetracycline is a thermostable member of the tetracycline group of antibiotics. Tetracyclines are bacteriostatic and inhibit multiplication of bacteria by reversible binding to the ribosome and blocking protein synthesis. Resistance to tetracyclines occurs mainly through efflux pumps, alteration of the ribosome to block binding of the antibiotic, or enzymatic inactivation of the antibiotic (Roberts, 2005). Tetracyclines, including oxytetracycline, exhibit cross-resistance and are considered a high-risk group for resistance development.

Oxytetracycline was registered for crop protection in the US in 1972, partially to provide an alternative antibiotic for fire blight management, especially for pear cultivated in regions with streptomycin-resistant populations of *E. amylovora*. Oxytetracycline also was registered to control a damaging disease of peaches and nectarines called bacterial spot caused by *Xanthomonas campestris* pv. *pruni*. As a crop pesticide, oxytetracycline is formulated as oxytetracycline-HCl or oxytetracycline calcium complex. For fire blight management, growers often combine of oxytetracycline with streptomycin and apply the materials as a 'tank mix.' Although, tetracyclines are considered high-risk for resistance development, resistance to field-doses of oxytetracycline has not been detected in field isolates of *E. amylovora* or *X. campestris pv. pruni*.

Oxytetracycline is applied at a dose of 150 ppm on peaches and nectarines for control of bacterial spot. The sprays begin at petal fall and can continue on 4 to 7 day intervals until 21 days before harvest. Up to nine applications of oxytetracycline are permitted annually on peach or nectarine.

For fire blight management, oxytetracycline is applied at 200 ppm on pear and apple. The applications can begin during early bloom and continue at 3 to 6 day intervals through bloom and weather conditions that favor the disease. Up to six applications of oxytetracycline are permitted on apple and up to 10 applications are permitted on pear annually. The preharvest interval for oxytetracycline on pear and apple is 60 days.

Figure 2 provides oxytetracycline use in orchards in the US from 1991 to 2015 with data summarized from the NASS QuickStats database. Usage of oxytetracycline were fairly consistent over 20 years, but increased in the last two reporting periods, when the acreage of apple treated increased and a greater number of applications were applied to peach in 2011 (Figure 2). In 2015, the NASS database reports that a total of 12,020 kg of oxytetracycline was applied to orchards (Table 3). The USGS estimated similar quantities, between 12,470 to 13,998 kg oxytetracycline in 2015.

In 2015, oxytetracycline was sprayed most frequently on pear, in part, due to the inherent sensitivity of pear to fire blight and the presence of streptomycin-resistant populations of *E. amylovora* in the western states of the US where pear is grown commercially (Loper *et al.*, 1991; Table 3). Similar to the observations on streptomycin use, the quantity of oxytetracycline used for plant protection in the US is much lower than the amounts permitted on the product labels.

Along with streptomycin, the US EPA granted emergency use registrations for oxytetracycline on citrus in Florida and California for management of the disease citrus greening. Usage data on oxytetracycline on citrus under these restricted emergency uses are not publically available at this time.

In addition to the US, oxytetracycline is permitted for crop protection in Latin America (Table 3). Oxytetracycline is packaged as a single antibiotic product or packaged as antimicrobial combinations of oxytetracycline plus streptomycin or oxytetracycline plus streptomycin and copper. These formulations are used for management of fire blight on pear in Mexico (Table 3). Oxytetracycline is also packaged and applied in combination with gentamicin and/or copper for management of diseases of flowers and vegetable crops in Latin America. It is not known how much oxytetracycline is applied to crops in countries in Latin America.

Kasugamycin (CAS 19408-46-9). Kasugamycin is a novel, structurally-unique aminoglycoside originally isolated from *Streptomyces kasugaensis* in Japan. Kasugamycin, also called kasumin, inhibits protein synthesis by a different mechanism than other aminoglycosides (Yoshii et al., 2012). Resistance to kasugamycin in plant pathogens occurs via spontaneous mutation in the *ksg* operon (dimethyltransferase) or 16SrRNA, or via modification by an acetyltransferase. Kasugamycin has no clinical or veterinary applications. There is no known cross-resistance between kasugamycin and aminoglycosides. Also, kasugamycin resistance is not known to be linked to resistance to antibiotics used in human medicine. For these reasons kasugamycin use as a pesticide is not considered a risk for the selection of resistance that affects human health. It is important to periodically monitor kasugamycin for cross-resistance and co-selection potential.

Gentamicin (CAS 1403-66-3). Gentamicin is an aminoglycoside used for control of several bacterial diseases of agave, vegetables, peppers, pear, rice, tomatoes and tobacco in countries in Latin America. It is also an antibiotic commonly used in human medicine, including treatment of serious bacterial infections. According to product labels, gentamicin is not sold as a single antimicrobial product, but rather in combination with oxytetracycline and/or copper compounds (Table 3). The labels for products

containing gentamicin were accessed on the website
www.terralia.com/agroquimicos_de_mexico/composition_index. For protection of crops, products
containing gentamicin are applied to fields between two to four times at seven day intervals. The re-
entry time into the treated areas often is listed as 12 hours after application. The pre-harvest interval
was not specified on labels consistently, expect for pear, which is between 21 to 30 days depending on
the product. Usage data on gentamicin in Latin American countries was not found.

Oxolinic acid (CAS 14698-29-4). Oxolinic acid is a synthetic quinolone that inhibits the enzyme DNA
gyrase. Oxolinic acid is related to fluoroquinolone antibiotics, which are commonly used in human
medicine. Oxolinic acid has been used in Israel to control fire blight on pear since 1998, after
streptomycin-resistant populations of *E. amylovora* emerged. The efficacy of oxolinic acid for fire blight
control on pear has decreased over time, in part due to resistance to the antibiotic (Kleitman *et al.* 2005,
Shtienberg *et al.*, 2015).   Oxolinic acid has been used in Japan and other countries for management of
bacterial diseases of rice (Hikichi *et al.* 1989, Maeda *et al.*, 2004). It is unclear how many countries
permit the use of oxolinic acid for disease management and which crops are treated.

Copper (CAS 7440-50-8). Copper is the most widely used compound for management of bacterial and
fungal diseases of plants. Copper containing crop pesticides are used on nearly every food crop, crops
grown for animal feed, and ornamentals. As a crop pesticide, copper can be phytotoxic and cause
damage to plants, especially on newly growing shoots and leaves. As a pesticide, there are concerns
about accumulation of copper in soils resulting in phytotoxicity. Copper also has been shown to co-
select for antimicrobial resistance. This subject has been widely reviewed (examples: see Baker-Austin
et al. 2006; Seiler and Beredonk, 2012; and Wales and Davies, 2015).

Copper underwent a re-registration review by the US EPA in 2009. The summary of the decision is
available at https://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/red_G-26_26-
May-09.pdf. At that time, product labels were amended to include methods reduce the potential for
spray drift to non-target areas for ground and aerial applications. The re-entry time for all copper-
containing pesticides was designated as 48 hours for field use and 24 hours for greenhouse use.
Additional statements related to potential environmental hazards, especially regarding toxicity to fish,
aquatic invertebrates, and aquatic systems, were added to labels. Finally, maximum amounts of copper
per application, reapplication intervals, and maximum annual rates of copper per acre were established
for all crops. The actual use rates for copper on crops are presented in Appendix A in the re-registration

document cited above. The annual maximum rates of the copper ion permitted on food crops vary greatly from 1.2 kg/HA for cereal grains to 53 kg/HA for mango.

Estimates on copper use in the US were obtained from the USGS database. The copper-component of crop pesticides varies among products. For example, copper may be included as metallic copper, copper hydroxide, copper octanoate, copper oxychoride, copper sulfate, or other forms. The usage data for copper-based pesticides are normalized to amount of the copper (active ingredient) present in the product. The total amount of copper used as crop pesticides was aggregated across formulations. Approximately 4,216,580 to 4,588,046 kg of copper was applied to plants in the US in 2015.

The data from the USGS represents commercial farm use of copper. Copper containing products sold for residential use for disease control on garden and landscape plants and moss control in lawns are widely available. Estimates on copper use by homeowners are not available.

Antifungal Chemicals

At least 36 triazole agricultural fungicides exist, although only a subset are currently used in any given country. Most triazole fungicides end with the suffix "-azole;" However, several triazoles do not (e.g., myclobutanil, triadimefon, and flutriafol), and a few fungicides with that suffix belong to other fungicide classes (e.g., imidazoles, benzimidizoles). Certain agricultural triazoles (i.e., bromuconazole, difenoconazole, epoxiconazole, propiconazole, and tebuconazole) docked more similarly on the *A. fumigatus* protein commonly involved in resistance to medical triazoles than did other triazole fungicides tested (e.g., triadimefon).

Across countries, the most detailed publicly available data on triazole agriculture use comes from the United States. According to the USGS Pesticide National Synthesis Project, which provides both low and high use estimates, total triazole use more than quintupled from ~350–600 metric tons in 1992 to ~2,600–3,750 metric tons in 2015 (preliminary estimates) (Figure). Use of two of the three triazoles with highest use in 1992 declined markedly over the following 23 years: triadimefon (131 to 0.09 metric tons; high estimates) and myclobutanil (129 to 46 metric tons) (Figure). In contrast, use of the most commonly used triazole in 1992, propiconazole, rose markedly (274 to 1,012 metric tons). Several triazoles introduced since 1992 were estimated to be the other most heavily used in 2015: tebuconazole (1,256 metric tons), prothioconazole (412 metric tons), metconazole (217 metric tons), and difenoconazole (176 metric tons).). In addition to triazoles applied in commercial agricultural settings by trained and certified applicators, myclobutanil, propiconazole, tebuconazole and triticonazole are

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available in products intended for home use for control of various fungal diseases of lawn and garden plants. Information on use of triazoles by homeowners is not available.

Data from other countries are available through the FAOSTAT website of the Food and Agriculture Organization of the United Nations based on questionnaires submitted by member countries. In these data, triazoles are grouped with imidazoles (also known as diazoles) and cannot be identified separately. In the United States, imidazole use was <1% that of triazole use in 2015 preliminary estimates, suggesting that combined triazole and imidazole use may be a reasonable proxy for triazole use, although these relative proportions likely differ in other countries. Of countries that reported data for 2014, Ukraine (2,996 metric tons), Germany (2,705 metric tons), France (2,241 metric tons), the United Kingdom (1,430 metric tons), and Poland (1,230 metric tons) had the highest reported use of triazoles and imidazoles. Triazole and imidazole use more than tripled between 2005 and 2014 in Poland and increased by 180% in Ukraine, 125% in the UK, and 70% in Germany (France did not report data in 2005). Further exploration of these data are needed, including adjustment for arable land area, particularly since Ukraine, Germany, and France reported triazole and imidazole use nearly as high as that of the United States, which has a far larger land area.

Other antimicrobial compounds used in plant protection

In countries in Asia, other synthetic antibiotics or antimicrobial natural products are used for crop protection. One example is Jingangmycin, which is validamycin A (CAS 37248-47-8) and synthesized by *Streptomyces* spp. Jingangmycin inhibits the enzyme trehalase (Shigemoto *et al.* 1992) and is used in Asia for control of sheath blight of rice caused by the fungal pathogen, *Rhizoctonia solani* (Kim, Y-S *et al.* 2015). Ningnanmycin (CAS 156410-09-2) is a synthetic pyrimidine nucleoside antibiotic with activity against viral diseases of plants and fungal diseases like powdery mildew (label information at http://www.cdxzy.com/en/proC/201209/156.html).

In this review, we acknowledge that there are many 'antimicrobial' materials that may be applied to crop plants in different countries. Little is known about the use of these materials for crop protection and apparently, these compounds do not have a putative link for resistance to clinical antimicrobials. Consequently, these compounds are beyond the scope of the review on antimicrobials used for crop protection.

Table 1. Antimicrobials used as pesticides

Antimicrobial/Pesticide	Relationship to Antimicrobials Used in Human Medicine	Cross-selection or Cross-resistance to Antimicrobials Used in Human Medicine
Oxytetracycline	A member of the tetracycline class of antimicrobials. These drugs are commonly used to treat infections caused by both Gram-negative and Gram-positive bacteria.	There are several resistance mechanisms that confer cross-resistance among the tetracycline antimicrobials.
Streptomycin	Streptomycin is used in human medicine and it is related to several other aminoglycosides (i.e., amikacin, gentamicin, tobramycin, plazomycin) that are commonly used to treat serious infections caused by both Gram-negative and Gram-positive bacteria.	Streptomycin can select for plasmid-mediated resistance mechanisms that confer resistance to all aminoglycosides.
Kasugamycin	Kasugamycin is not used in human medicine and it is structurally dissimilar to related drugs, aminoglycosides, that are used in human medicine.	Kasugamycin resistance mechanisms do not select for resistance to aminoglycosides used in human medicine and resistance to aminoglycosides used in human medicine do not confer resistance to kasugamycin. There is no evidence for cross-resistance. There is also no evidence for co-selection.
Oxolinic Acid	Oxolinic acid is a quinolone and is related to fluoroquinolones commonly used in human medicine, like ciprofloxacin and levofloxacin.	Quinolone resistance confers cross-resistance to fluoroquinolones (Barry, 1984).
Copper	Copper is heavy metal and unrelated to antimicrobials used in human medicine.	Disease causing bacteria can carry heavy metal resistance in plasmids (mobile genetic elements) along with resistance to antibiotics used in human medicine. Therefore, copper has co-selection potential.
Triazoles	Triazoles are a class of fungicide that are related to azole antifungals, like fluconazole and intraconazole, commonly used to treat human fungal infections.	Cross-resistance between triazoles and azoles used in human medicine occurs.

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Table 2. Registered uses of streptomycin for crop protection in the USA

Crop	Disease (causal agent)	Provisions
Tree fruit		
Apple	Fire blight (*Erwinia amylovora*)	Begin 100 ppm sprays at early to full bloom, then every 4 to 7 days during bloom. Continue sprays every 7 to 14 days until 50 days before harvest. May apply 6 to 8 times after bloom.
Pear	Fire blight (*E. amylovora*)	Begin 100 ppm sprays at early bloom, then every 3 to 5 days during bloom. Continue sprays every 5 to 14 days until 30 days before harvest. May apply up to 15 times during the season.
Seedlings grown in greenhouses until transplanted to field		
Celery (Florida only)	Bacterial blight (*Pseudomonas cichorii*)	Apply at 200 ppm. First application at two-leaf stage, then at 4 to 5 day intervals until celery is transplanted in field.
Peppers and tomato	Bacterial spot (*Xanthomonas euvesicatoria* and *Xanthomonas perforans*) Bacterial Speck (*Pseudomonas syringae* pv. tomato)	Apply at 200 ppm. First application at two-leaf stage, then at 4 to 5 day intervals until transplanted in field.
Row Crops		
Potato	Soft rot black leg (*Pectobacterium* spp.)	Soak cut seed pieces in 100 ppm streptomycin for several minutes, then plant in field.
Tobacco	Blue mold (*Peronospora tabacina*) Wildfire (*Pseudomonas syringae* pv. *tabaci*)	Apply at 100 or 200 ppm when plants are in two-leaf stage or when disease appears. Repeat at 5 to 7 day intervals until plants establish in field. Option to continue applications at weekly intervals.
Ornamentals		
Apple, Cotoneaster, Pear, Pyracantha	Fire blight (*E. amylovora*)	Apply at 100 ppm in early bloom, then every 3 to 4 days. After bloom spray every 5 to 7 days until fruit are visible.
Cuttings: Chrysanthemum, Dieffenbachia	Bacterial wilt Bacterial stem rot (*Erwinia* spp. and *Pseudomonas* spp.)	Soak cuttings in 50 ppm or 200 ppm streptomycin for 4 hours or 20 minutes, respectively. Plant in sterile potting medium.
Numerous plants: e.g. Carnation, Forsythia, Lilac, Philodendron	Bacterial leaf rot (*Xanthomonas campestris*)	Apply at 200 ppm every 4 to 5 days. If symptoms present, remove rotted leaves and spray every 4 days.
Roses (New Jersey only)	Crown gall (*Agrobacterium* spp.)	Remove galled tissue, soak root system and cut surfaces of plant in 200 ppm streptomycin for 15 minutes and replant in clean soil.

Table 3. Antimicrobials used as crop pesticides in countries in Latin America

Crop	Disease	Causal agent	Materials§						
			Gm + oTc	Gm + oTc + Cu	oTc	oTc + Cu	oTc + Sm	oTc + Sm + Cu	Sm
Agave	Soft rot	*Pectobacterium carotovora*	X¥	X	-	-	-	-	-
Apple	Fire blight	*Erwinia amylovora*	-	-	-	-	-	-	X
Asparagus, garlic, onion, scallion	Bulb rot and Bacterial blight	*Pseudomonas viridiflava* and *Xanthomonas campestris*	X	-	-	-	-	-	-
Carnation	Bacterial spot	*Burkholderia andropogonis*	X	-	-	X	-	-	-
Celery	Bacterial blight	*Pseudomonas cichorii*	-	-	-	-	-	-	X*
Chrysanthemum	Soft rot	*Erwinia chrysanthemi* pv. *chrysanthemi*	X	-	-	X	-	-	-
Cucumber, melons, and squash	Angular leaf spot and rot	*Pseudomonas syringae* pv. *lachrymans* and *Acidovorax avenae*	X	X	-	X	-	-	-
Eggplant, chili, peppers, potato, tomato, and tomatillo	Bacterial leaf spot	*Xanthomonas campestris* pv. *vesicatoria* and *Ralstonia solanacearum*	X	X	-	X	-	-	X*
Ornamentals	Crown gall and fire blight	*Agrobacterium* spp. and *E. amylovora*	-	-	-	-	-	-	X
Pear	Fire blight	*Erwinia amylovora*	X	-	X	-	X	X	-
Potato	Black leg and bacterial wilt	*Erwinia carotovora* spp. *atroseptica* and *Ralstonia solanacearum*	X	-	-	-	-	-	X*
Rice	Bacterial blight	*Xanthomonas campestris* pv. *oryzae*	X	-	-	-	-	-	-
Tobacco	Bacterial wilt and wildfire	*Ralsonia solanacearum* and *Pseudomonas syringae* pv. *tabaci*	X	-	-	X	-	-	-

§ Single antimicrobials and packaged mixtures. Cu= copper, Gm=gentamicin, oTc=oxytetracycline, and Sm=streptomycin.

¥ X indicates material used on crop; - denotes material not listed for crop.

* Indicates application only to seed or tubers.

Table 4. Current registered uses of kasugamycin in Canada, New Zealand, and the United States

| Crop (country)	Disease/causal agent	Provisions
Cherry trees (USA)	Bacterial blast and Bacterial canker (*Pseudomonas syringae* pv. *syringae*)	No more than 4 applications per year. Minimum interval between applications is 7 days. No more than two consecutive applications. No applications after petal fall or within 30 days before harvest.
Fruiting vegetables: e.g. eggplant, peppers, tomatillo, tomato. (Canada)	Bacterial spot (*Xanthomonas campestris* pv *vesicatoria*) and Bacterial stem canker (*Clavibacter michiganensis* spp *michiganensis*)	No more than 3 applications per year. Minimum interval between applications is 7 days. No more than two consecutive applications. Do not use on greenhouse transplants. No applications of Kasumin within 1 day before harvest.
Kiwifruit vines (New Zealand)	Psa (*Pseudomonas syringae* pv. *actinidiae*)	4 applications maximum per year, until 21 days before bloom. No more than two sequential applications, rotate with other materials, e.g. copper or streptomycin
Pome fruit trees e.g. apple and pear (Canada and USA)	Fire blight (*Erwinia amylovora*)	No more than 4 applications per year, beginning at 20-30% bloom. Minimum interval between applications is 3 to 7 days. No more than two consecutive applications. No applications within 90 days of harvest.
Walnut trees (USA)	Walnut blight (*Xanthomonas campestris* pv. *juglandis*)	No more than 4 applications per year. Minimum interval between applications is 7 to 14 days. No more than two consecutive applications. No applications within 100 days of harvest.

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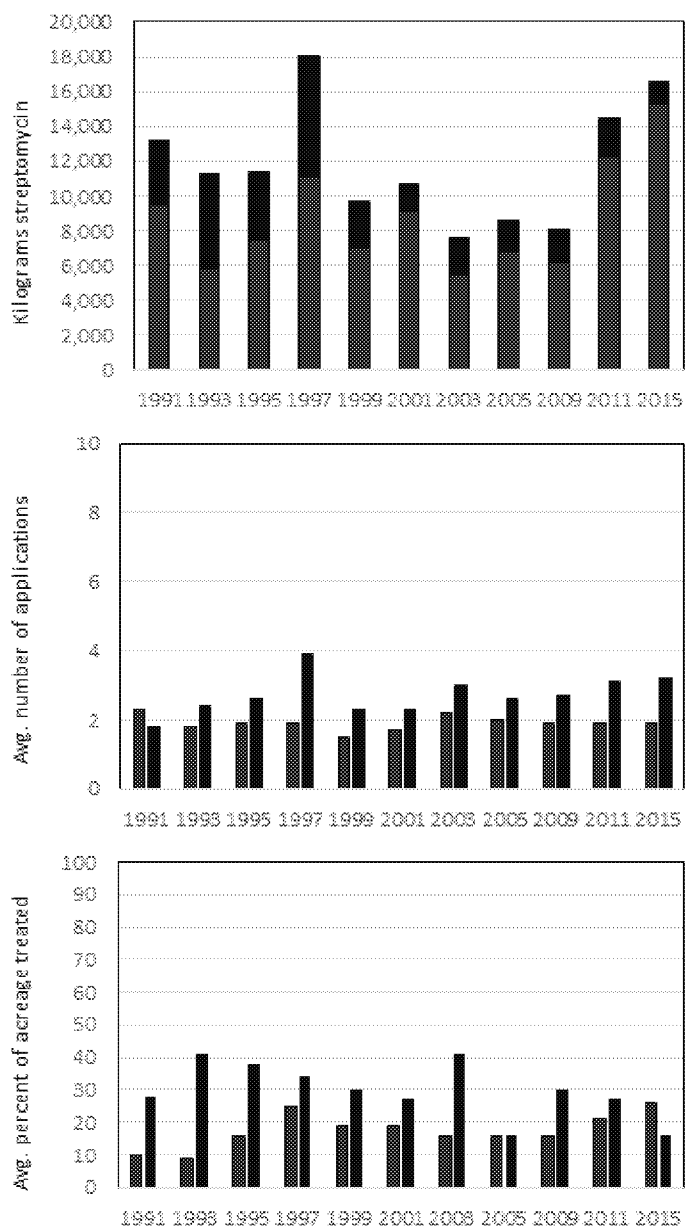
Figure 1. Usage of streptomycin on apple (red bars) and pear (black bars) in the US from 1991 to 2015. Usage data was obtained from the USDA National Agricultural Statistics Service QuickStats database. The upper graph is the total quantity of streptomycin in kilograms applied annually. The middle graph depicts the average number of applications of streptomycin on crops. The bottom graph shows the average percent of total acreage of a crop that was treated with streptomycin at least once.
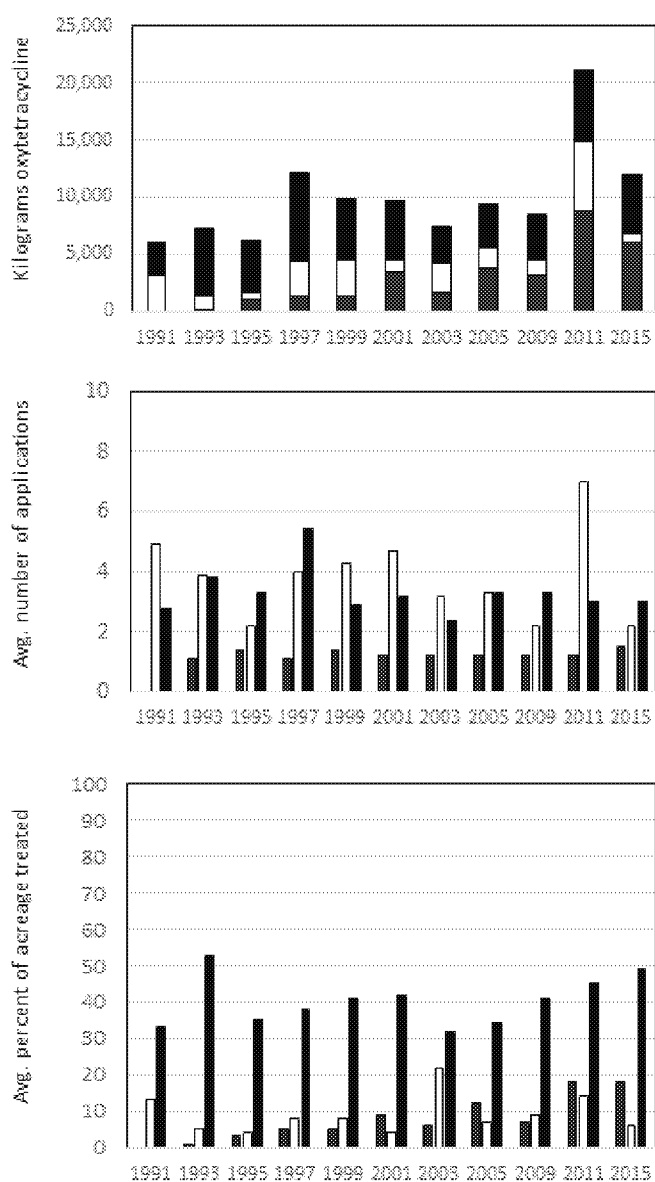
Figure 2. Usage of oxytetracycline on apple (red bars), peach (white bars), and pear (black bars) in the U.S. from 1991 to 2015. Usage data was obtained from the USDA National Agricultural Statistics Service QuickStats database. The upper graph is the total quantity of oxytetracycline in kilograms applied annually. The middle graph depicts the average number of applications of oxytetracycline on crops. The bottom graph shows the average percent of total acreage of a crop treated with oxytetracycline at least once.
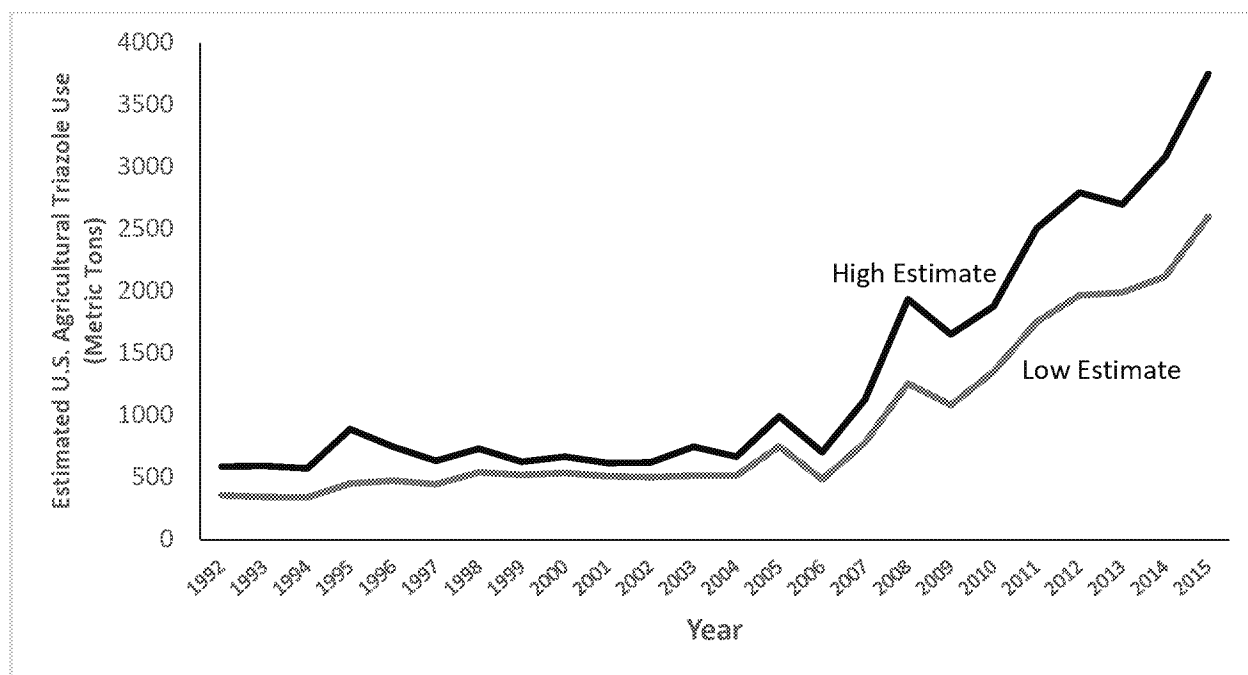
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Figure 3. Low and high estimates of U.S. agricultural triazole fungicide use by year. Data for 2013–2015 are preliminary estimates that may be revised based on updated crop acreage data. Data from 2015 do not include estimates for seed treatment applications. Source: United States Geological Survey Pesticide National Synthesis Project (https://water.usgs.gov/nawqa/pnsp/usage/maps/county-level/).
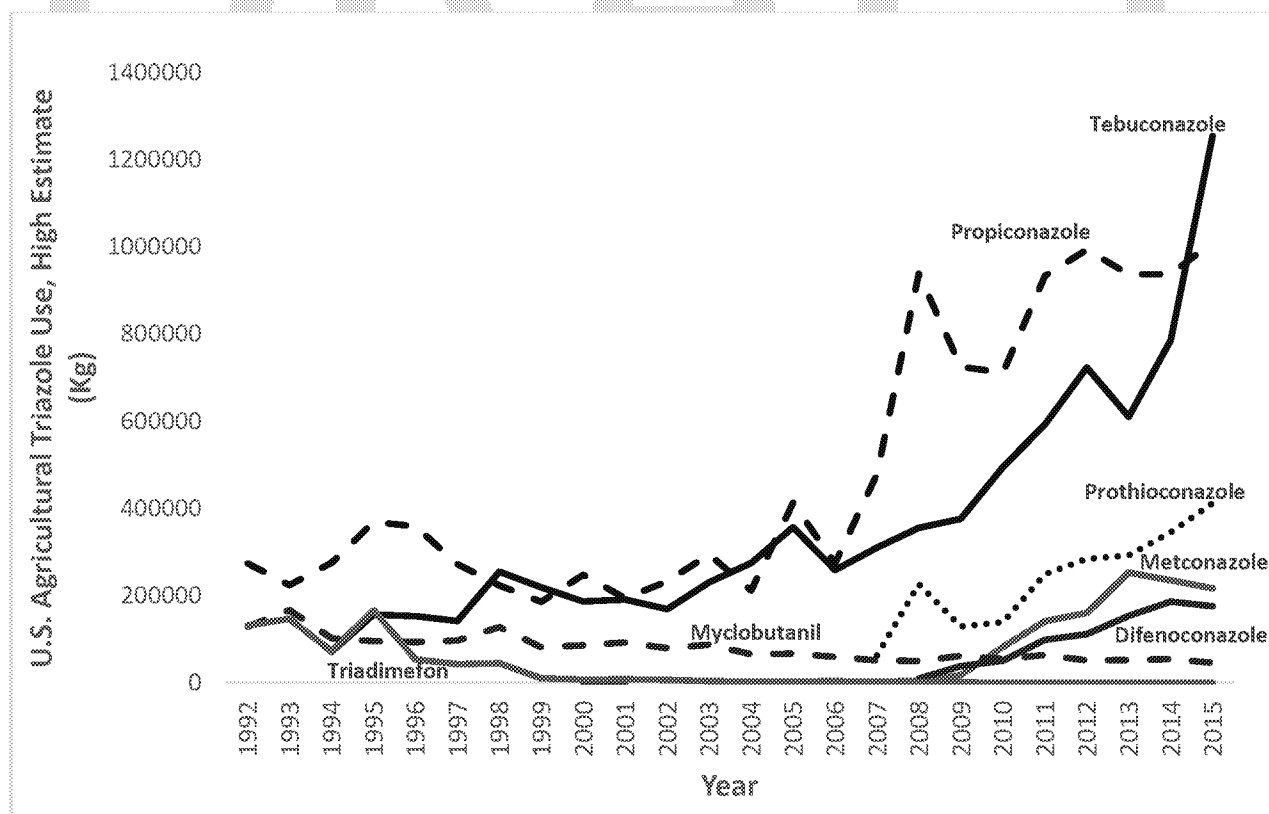
Figure 4. Agricultural fungicide use (high estimates) in the United States by year for all three triazole fungicides used in 1992 and the 5 most heavily used triazoles in 2015. Source: United States Geological Survey Pesticide National Synthesis Project (https://water.usgs.gov/nawqa/pnsp/usage/maps/county-level/).
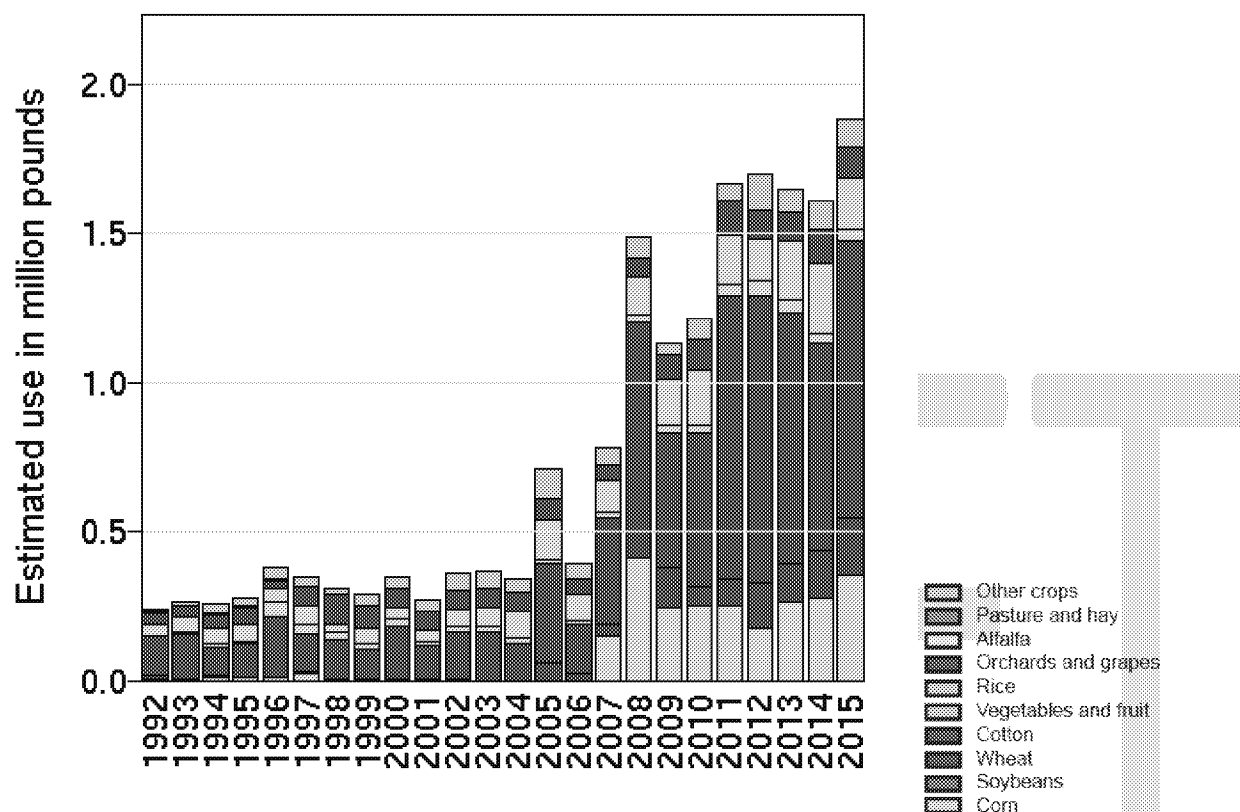

Figure 5. U.S. agricultural tebuconazole use (high estimates) by year and crop. Source: United States Geological Survey Pesticide National Synthesis Project (https://water.usgs.gov/nawqa/pnsp/usage/maps/county-level/).

B. When antimicrobials are used as pesticides, what is the exposure of personnel applying the pesticide? What is the exposure of personnel working on the farm after exposure? What is the risk from this exposure?

Use of plant protection products (PPPs) can lead to exposure of microbes, both bacteria and fungi, associated with humans, animals (including pets) and the environment. PPPs are routinely tested for toxicity to humans by studying toxicity in experimental animals. However this testing does not include measuring the antimicrobial effect of the PPP but not for sub-lethal effects on microbes, so little is known about possible effects of PPPs on the human microbiome disruption.

In Europe, use of PPPs in agricultural use is regulated and Acceptable Daily Intakes (ADI) and Acute Reference Doses (ARfD) for consumers are set by regulatory authorities like EFSA. Acceptable Daily Intakeor ADI is a measure of the amount of a specific substance (originally applied for a food additive, later also for a residue of a veterinary drug or pesticide) in food or drinking water that can be ingested (orally) on a daily basis over a lifetime without an appreciable health risk. ADIs are expressed usually in milligrams (of the substance) per kilograms of body weight per day. Acceptable Operator Exposure Level (AOEL) are defined for operators handling the PPPs. Both ADIs and ARfD values are based on No-Adverse effect levels (NoAEL) in toxicology studies in animals studies divided by a safety factor. This safety factor is conventionally set at 100, to account for the differences between test animals and humans (factor of 10) and possible differences in sensitivity between humans (another factor of 10). ADI, ArfD and AOIL values as set by EFSA for some PPPs with potential antimicrobial activity are given in Table 5. Please note that potential anti-microbiological properties are not taken into account when setting these values since these properties are not routinely studied

In Europe, EFSA (2014) has issued guidance to assess exposure of operators, workers, residents and bystanders to PPPs and this guidance is based on a large number of databases and scenarios. Possible exposure routes are inhalation, dermal and oral.

It is important to separate the exposed groups to PPPs in different categories:
Operators are involved in activities relating to the application of a PPP including mixing and loading. Operators should use suitable personal protective equipment. Workers are persons who, as part of their employment, enter an area that has been treated previously with PPP or who handle a crop that has been treated with a PPP. Bystanders are persons who could be located within or directly adjacent to the area where the PPP was applied and their presence was unrelated to work with PPPs. Bystanders are often acutely exposed and have not used personal protective equipment.
Residents are persons who live, work or attend school of any other institution adjacent to an area that is or has been treated with a PPP. Residents can have a long-term exposure and have not used personal protective equipment. Exposure to PPPs can be by inhalation, oral or dermal. Different models and approaches are available (see EFSA, 2014), but it was noted that the dataset for assessing resident and bystander exposure is rather limited. Exposure is also dependent on the formulation type of the PPP (for example powder vs aerosols). In addition, it should be assessed if the PPP has potential for systemic toxicity from exposure during a single day.

Biomonitoring of workers handling PPPs might give more realistic exposure data, especially if the compounds or metabolites are measured in blood or serum, but data on relevant compounds is scarce in the scientific literature. Probably more data is to be found in the gray literature.

It should be noted that some PPPs are also used in in the domestic setting in private gardens. This kind of exposure is not regulated and depends that consumers read and follow the instructions of use for that particular PPPs.

In Table 5 ADI, ARfD and AOEL values as set by EFSA for some selected PPPs associated with potential antibiotic potential are given.

The data presented in Table 1 indicates that no antimicrobial agents classes (aminoglycosides, tetracyclines, and quinolones) with antibacterial activity, are approved as PPPs in Europe.

Table 5 - ADI, ARfD and AOEL values (as set by EFSA) for some selected PPPs associated with potential antibiotic potential.

Aminoglycosides			ADI	ARfD	AOEL
			mg/kg bw per day	mg/kg bw	mg/kg bw per day
Streptomycin	57-92-1	Not approved in europe			
Gentamycin	1403-66-3	Not approved in europe			
Kasugamycin	19408-46-9	Not approved in europe			
Validamycin A	37248-47-8	Not approved in europe			
Tetracyclines					
Oxytetracycline	79-57-2	Not approved in Europe			
Quinolones					
Oxolinic acid	14698-29-4	Not approved in Europe			
Triazoles					
Propiconazole	60207-90-1	AT, BE, BG, CY, CZ, D	0.04	0.3	0.1
Tebuconazole	107534-96-3	AT, BE, BG, CY, CZ, D	0.03	0.03	0.03
Epoxiconazole	13855-98-8	AT, BE, BG, CZ, DE, C	0.008	0.023	0.008
Difenoconazole	119446-68-3	AT, BE, BG, CY, CZ, D	0.01	0.16	0.16
Bromuconazole	116255-48-2	AT, BE, CZ, DE, ES, FI	0.01	0.1	0.025

C. To what extent do antimicrobials used as pesticides contaminate the environment surrounding the crop field? What measure are effective in limiting spread?

Examples in which antimicrobials used as pesticides have been detected in the environment surrounding the crop field

While antibiotics are now extensively monitored in many environmental compartments, data specific to antimicrobials used as pesticides are scarce. For instance, oxytetracycline (OTC) is frequently detected in aquatic systems such as intensively managed agricultural watershed (Dungan, 2017). However, OTC in the environment is largely associated with its widespread use in veterinary drug. To date, no experimental data links OTC occurrence in nature to its use as a pesticide. Similar conclusions may be drawn for oxolinic acid and aminoglycoside antibiotics. With regards to triazole fungicides, propiconazole and tebuconazole were detected in streams across the U.S. (Battaglin, 2011). These antifungals are widely used in agriculture, and their occurrence was suggested to be related to their use in upstream areas. Maximum concentration of propiconazole at sampling sites was correlated with estimates of the antifungal use in upstream drainage basins. Propiconazole and tebuconazole were also detected in surface waters in Switzerland (Kahle, 2008), which was suspected, though not confirmed, to originate from agricultural use or urban runoff rainwater. In another study, tebuconazole was detected in sediment and amphibian tissue samples from Yosemite National Park and other sites in California's Sierra Nevada mountains (Smalling, 2013). Because this fungicide was not known to be used at those sites, but was heavily used in the downwind agricultural Central Valley, the researchers suspected airborne deposition. Overall, few studies have examined occurrence of triazoles in the environment despite a substantial increase in use in the U.S. since 2005 (https://water.usgs.gov/nawqa/pnsp/usage/maps/compound_listing.php).

Ecological and human health risk assessments have relied mostly on predicting environmental concentrations based on modelling. For example, the US EPA calculated upper bounds concentrations of streptomycin or OTC that might be found in surface and ground waters due to their use on apple (aerial spray application scenario) (US EPA streptomycin, 2006) or peach/nectarine orchards respectively (US EPA oxytetracycline, 2006). Modelling was also applied to obtain the worst case global maximum epoxiconazole concentration (1.215 mg/L) for the stream runoffs (Chambers, 2014). A model of triazole use on soybeans estimated that these antifungals would be present in field runoff and shallow groundwater in concentrations that exceed chronic human health exposure thresholds (Deb, 2010).

Parameters influencing the mobility of antimicrobials in the environment

The environmental fate of a pesticide is influenced by factors such as their physicochemical properties, their mode of application, soil and hydrological conditions, or climatic conditions. Compounds such as OTC and aminoglycosides are quite water soluble (Royal Society of Chemistry, 2017), whereas triazoles are relatively less so. This range suggests differences in terms of mobility and fate in the environment. With regards to the different modes of pesticide application; for example when applied as spray, simulated heavy rainfalls removed OTC from the leaf surface within minutes (Christiano, 2010). However, when injected into the trunk of citrus trees, OTC residues may persist in the leaves and roots for months (Hu, 2016).

Soil characteristics (including pH, ionic strength, metal ions, and organic matter content) influence the adsorption processes of antibiotics and their mobility (Wang, 2015; ter Laak, 2006). Recent studies seem to indicate that even though a compound may be adsorbed by soil it may still exert selective pressure on exposed bacteria, increasing risk that resistance might be developed (Chen, 2017). A better understanding of selective pressure of antimicrobials in soil systems is still needed (Gonsalves, 1977).

Antimicrobial persistence in the environment

Abiotic degradation, biotic degradation and field dissipation studies are key information to understand the persistence and the fate of pesticides in the environment. Compounds such as validamycin A may dissipate relatively quickly in soil, as illustrated in a controlled conditions study where residues became undetectable after 7 days of spray application (Xu, 2009). Other compounds may be more persistent. For example, OTC residues could still be detected in low concentrations in soil after one and a half years of their last application (Gonsalves, 1977). Based on the monitoring data in lakes, Kahle et al. (2009), also suggested that triazole compounds (fluconazole, propiconazole, and tebuconazole) may be relatively persistent in the aquatic environment.

Hydrolysis and photolysis are major mechanisms of abiotic degradation, and environmental factors (e.g. light exposure, pH, and temperature) may influence their degradation (Christiano, 2010; Shen, 2017). Natural organic matter may also play a role in the fate of these compounds. For example, sorption on natural organic matter was shown to enhance phototransformation of aminoglycosides (Li, 2016).

It is important to mention that a disappearance of the parent compound does not correspond to a loss of antimicrobial activity. For example, the degradation products of streptomycin were shown to exhibit residual antimicrobial activity (Shen, 2017). In fact, the metabolites and degradation products of most antimicrobials have not yet been completely identified; hence their impact on AMR remains mostly unknown. Indeed, progresses in the field of mass spectrometry only recently allowed for the identification of metabolites in crops (Bauer, 2018) and the environment.

Limiting the spread of antimicrobials

Pesticide product labels contain general recommendations from the suppliers, such as (i) not applying directly to water, to areas where surface water is present, or to intertidal areas below the mean high water mark. Labels warn that the usage of some of these chemicals may result in groundwater contamination in areas where soils are permeable, particularly where the water table is shallow. Recommendations also include (ii) not discharging equipment wash water or rinsate; (iii) not to apply when environmental conditions (e.g. wind) favor drift beyond the target application area, (iv) not exceed a maximum numbers of applications per season, and (v) preventing livestock to graze within the treated area (https://www.cropscience.bayer.us/products/fungicides/stratego/label-msds). However, in the absence of detailed monitoring data, it is difficult to assess whether these measures limit effectively the spread of the parent compounds or their metabolites and degradation products.

D. To what extent do antimicrobials select for resistance within the crop field or surrounding environments? Is this resistance a threat to human health?

General Principles for Evaluating Risk from Current Crop Uses of Antimicrobials

Selection for resistance is primarily a function <u>length of time</u> of exposure and <u>concentration</u> of chemical that the microbial populations experience. Other factors that contribute to probability of resistance selection are the microbial population size (since emergence or natural occurrence of resistance is a low frequency event) resources for growth to fix and amplify the resistance trait, and ease of occurrence of the resistance enabling trait.

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For the primary factors, time of exposure is determined by the frequency of use and the stability of the chemical in the microbe's habitat, often expressed as half-life. Dissipation of the antibiotic can result from biodegradation by (resistant) microbes, photochemical transformation or chemical hydrolysis, loss by volatilization or co-distillation to the atmosphere, leaching away, and dilution by water. Most antibiotics have a very low vapor pressure, so the loss by volatilization could be negligible.

The concentrations the microbes experience are also determined by the chemicals' bioavailability to the microbe, i.e., the amount that enters the cell and affects its critical functions. Bioavailability of many antibiotics is reduced in soil due to their sorption to soil particles or organic matter, hence reducing the selection for resistance. Subinhibitory concentrations – those that are below the level capable of inhibiting microbe growth and replication - however, can have other effects including inducing horizontal gene transfer which can confer resistance (Andersson and Hughes 2014; Davies et.al, 2006). Many of these time and concentration parameters are reported but can be estimated (and checked for consistency) from the basic properties of the antibiotic molecules, e.g. as summarized in Table 5.

The site of application can also have a substantial effect on resistance selection. If the application is to leaves and fruits, which is the case for most uses of these antibiotics, the microbe exposure is relatively low due to the lower microbial density in these habitats, and the higher potential for photochemical dissipation. Some are injected into tree trunks for which microbial exposure is very low. In a relative sense, these crop uses of antibiotics would be predicted to incur much less resistance selection than when antibiotic containing manures or recycled animal or urban waters are applied to soil. In contrast to the antibiotics, triazole fungicides are applied broadly, including by aerial and ground spray application, and in large quantities.

The selection of resistance in the environment depends upon which types of microorganisms are present and the density of these organism. It is common for environmental microorganisms to contain naturally occurring resistance mechanisms. The presence of an antimicrobial in the environment could result in the amplification of these resistant environmental bacteria. It is also possible for resistant genes in these bacteria to be mobilized into transferable genetic elements like plasmids. These mobile elements allow for resistance to move from one bacteria to another; a process also known as horizontal gene transfer. For horizontal gene transfer to occur among bacteria the following are necessary: the antibiotic resistance trait is on a mobile genetic element, high density of genetically related organisms are present (since cell-cell contact and genetic compatibility are necessary), and there is an available carbon source for the cell to complete its growth functions. Horizontal gene transfer of antibiotic

resistance traits to a pathogen or to a commensal that co-inhabits environments with a human pathogen provide the highest risk scenarios. Another scenario is the presence of human pathogens with mobile genetic elements in the environment from contamination of human waste or animal waste. In this case the presence of the antimicrobial could result in the amplification of the resistant human pathogen. The crop use of antibiotics would seem to provide negligible risk for this scenario of HGT, but monitoring is needed, especially in when the environment is contaminated with human pathogens.

Antibiotics used in agricultural plants

Antibiotics have been commonly applied to control the fire blight pathogen *Erwinia amylovora* located in apple or pear flowers. The *E. amylovora* populations quickly grow on flower stigmas under favorable weathers, and could be further disseminated among flowers by pollinators. Blossom blight infection results from the bacterial entrance into the flower, which could cause the death of flowering spur.

Currently in the United States, streptomycin, kasugamicin and oxytetracycline have been registered for fire blight management during bloom season. These antibiotics are commonly applied by air blast spray to apple and pear trees. This spray technique has a relatively low delivery rates to target, and could cause varying fractions of antibiotics to drift into the soil environment. The application of pesticides in a similar manner demonstrates approximately 25-71% of loss to the surrounding soils (Moltó et al., 2001; Rumker et al., 1975; Steiner, 1969). The antibiotic concentration in the spray is suggested to be in the range of several hundreds of milligrams per liter. For instance, oxytetracycline is recommended to apply at 200 mg/L in non-ionic surfactant Regulaid solution (Sundin, 2015). If one liter of spray is applied to a tree with a canopy radius of 2 m, and 50% of the applied oxytetracycline drifts to the environment, the concentration of oxytetracycline depositing to the surrounding soils could reach > 100 µg/kg (ppb). Since oxytetracycline is strongly sorbed by soils with sorption coefficient of 420-1030 L/kg, and the half-life is relatively long i.e. 25-56 days, ppb levels of oxytetracycline in soils could last for several months, which provides sufficient time for bacterial selection of antibiotic resistance. This level of oxytetracycline in soil, however, is below the minimum inhibitory concentration (MIC), suggesting that the antibiotic would not inhibit bacterial growth. Nonetheless, soil-sorbed oxytetracycline serves as a reservoir, which could continuously release oxytetracycline to soil pore water. The antimicrobial present in soil pore water could exert selection on the indigenous microbial strains to promote the development of antibiotic resistance or other antibiotic induced effects (Zhang et al., 2014).

Neutrally-charged tetracycline species posed much higher selective pressure on bacteria, compared to tetracycline complexed with metal cations and dissolved organic matter (Zhang et al., 2014).

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Oxytetracycline for spray application are sold as complexes, specifically, oxytetracycline-calcium complex and oxytetracycline-hydrochloride. The oxytetracycline-hydrochloride seems to demonstrate greater antimicrobial activity because it performs better than the oxytetracycline-calcium complex for blossom blight control in head-to-head comparisons (Sundin, 2015).

Besides the tetracycline present in soil water, soil-bound tetracyclines also pose selection on the bacteria attached to the same soil surfaces (Chen et al., 2017). In fact, levels of antibiotics below the Minimum Inhibitory Concentration (MIC) could cause even greater selection to microbial communities than those with antibiotic concentration more than the MIC, because of the larger bacterial populations on soil particle surfaces. In addition to the parent compound, the major degradation products of oxytetracycline are 4-epi-oxytetracycline, α-, β-apo-oxytetracycline (Halling-Sørensen et al., 2003), and these degradation products still contain the chemical functional moiety of antimicrobial activity and can pose additional selection on soil bacteria.

The antimicrobials used on crops (streptomycin, gentamicin, kasugamicin, validamycin A, oxytetracycline, oxytetracycline and oxolinic acid), are non-volatile with the vapor pressure < 10^{-7} mm Hg, suggesting that they are unlikely to expose bacteria by vapor phase. The water solubilities of these antimicrobials are high (> 1 g/L), except oxolinic acid (3.2 mg/L). Compared to oxytetracycline, these antibiotics demonstrate weaker affinity to soils and shorter half-lives (Table 5). This suggests that they may not persist long in soils and hence pose less selection on bacteria. Unfortunately, there are no data of the minimum concentration to invoke selection on bacteria. The MIC of validamycin A is 0.01 μg/mL which is a plausible level for some selection but its very short half-life (< 2 days) makes it unlikely to induce development of antibiotic resistance.

Triazole and Their Agricultural Uses

The half-lives of these triazole fungicides in soils range from 56-679 days, indicating that some of these antifungals could persist in soils at the levels of μg/kg from months to years. Triazole were found in agricultural lands at concentration of ~9 μg/L in water, and the associated concentration in soils is estimated at hundreds of μg/kg or even higher (Komárek et al., 2010). The relatively strong sorption by soils (K_{oc} 456-11202 L/kg) indicates that these chemicals should remain primarily in the root zone of soils

where microbial organisms are more numerous and active. These levels of fungicides (µg/kg) in soils could be desorbed into soil pore water, but the concentration could not reach the MIC levels (Table 2).

However, like the antibiotics present in soils, this level of antifunfal does provide some continuous selective pressure on soil fungi to develop resistance. The sub-MIC levels (µg/kg) and long persistence of these fungicides in soils create favorable concentration range for long-term selection for resistance. However, the aging process of these antifungals in soils could lessen their bioavailability to soil microorganisms, reducing the potential risks to invoke resistance to antifungals.

Resistance of *A. fumigatus*, a fungus widely present in natural and agricultural environments, to medically approved triazoles has become a major concern. This fungus thrives on decaying vegetation, particularly self-heating compost piles, where its ability to tolerate heat more than most other saprophytic fungi allows it to grow in large quantities, sometimes close to pure culture (Kwon-Chung, 2013). Cross-resistance between agricultural and medical triazole fungicides would not be surprising due to a common mode of action and seems to have occurred (Ribas et.al. 2016). These authors also report that other fungi have become resistant to this antifungal class and that some of these are becoming medically important. The resistances that have developed have been limited to a few genes mutations common among different strains and even genera (Ribas et. al. 2016). Horizontal gene transfer likely does not play a role in the environmental spread of triazole-resistant *A. fumigatus*. Instead, sexual and asexual reproduction of resistant strains and airborne spread is thought to be the route of transmission to humans (Berger 2017).

The threat of resistance to human health

Antibiotics and antifungals should be considered separately because there are chemical distinct and target microorganisms are distinct. For the antibiotics, their very limited and special uses, apple and pear, application to low density microbial habitats and low bioavailability would argue against probabilities for significant resistance selection. Their stability would be a factor counter to this summary, but overall, the risk to human health should be very low, and certainly so compared to the many other (non crop) environmental sources for antibiotic resistance selection. For the triazoles, the much larger, longer and more diverse uses and their stability in the environment would argue for much greater chances for resistance selection, which evidence supports. At present, the concern for antifungal

resistance from agricultural fungicide use is largely restricted to *A. fumigatus*, but much remains unknown about fungal pathogens, and more research is needed to understand the role of fungicides in contributing to resistance in other medically important fungi. For example, the yeast *Candida auris*, most known strains of which are resistant to the triazole fluconazole, has rapidly emerged as an important fungal disease in several world regions only in the last few years, and concern exists that agricultural triazole use may be affecting populations in related yeasts.

Table 6. Physical and chemical properties of antibiotics and fungicides used on crops

Chemical	CAS Number	K_d (L/kg)	K_{oc} (L/kg)	Half-life (day)	Water solubility mg/L	log K_{ow}	Vapor pressure (mm Hg)	MIC$_{50}$ (μg/mL)
Streptomycin	57-92-1	N/A	10[a]	17.5-25[a]	12800[b]	-7.53[b]	1.18×10^{-10} [m]	*Escherichia coli*: 1.56[c]
Gentamicin	1403-66-3	N/A	N/A	N/A	12600[b]	-1.88[b]	1.50×10^{-9} [m]	*Escherichia coli*: 0.5[d]
Kasugamycin	19408-46-9	345[e]	10-364[e]	42.8-73[e]	228000[e]	-1.96[e]	8.35×10^{-12} [m]	*Escherichia coli*: 500[f]
Validamycin A	37248-47-8	N/A	N/A	0.21[g], 1.5[y]	610000[b]	-8.32[b]	1.24×10^{-10} [m]	*Pellicularia sasakii*: 0.01[h]
Oxytetracycline	79-57-2	420-1030[i]	27800-93300[i]	25-56[j]	1000[b]	-1.22[b]	1.24×10^{-10} [m]	*Escherichia coli*: 1[k]
Oxolinic acid	14698-29-4	70-116[i]	1190-4510[i]	25.2[l]	3.2[m]	0.94[m]	3.63×10^{-8}[m]	*Escherichia coli*: 0.06[n]
Propiconazole	60207-90-1	N/A	1820[p]	55.8–365[q]	100[b]	3.72[b]	1.0×10^{-6}[b]	*Aspergillus fumigatus* :2-8[o]
Tebuconazole	107534-96-3	N/A	906-1251[r]	86-223[s]	36[b]	3.7[b]	1.3×10^{-8}[b]	*Aspergillus fumigatus* : 1-8[o]
Epoxiconazole	133855-98-8	N/A	456-1504[t]	120-354[u]	8.42[v]	3.58[v]	4.5×10^{-7}[v]	*Aspergillus fumigatus* : 2-16[o]
Difenoconazole	119446-68-3	N/A	3870-11202[w]	82.9-462[w]	15[b]	4.4[b]	2.5×10^{-10}[b]	*Aspergillus fumigatus* : 1-4[o]
Bromuconazole	116255-48-2	N/A	872[x]	123-679[x]	50[b]	3.24[b]	3.0×10^{-8}[b]	*Aspergillus fumigatus* : 1-4[o]

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E. How should environmental contamination of antimicrobials and emerging resistant bacteria be monitored?

Ongoing monitoring data in several areas are needed to address possible links between use of antimicrobial agents (i.e., selected antibacterials and triazoles) in agriculture and emergence of antimicrobial-resistant human pathogens.

1. **Pesticide use data**: Publicly available data on use of selected antibacterial agents and triazole fungicides in crop agriculture would allow researchers to target studies of antimicrobial resistance and evaluate geographic and temporal relationships between pesticide use and resistance. For many countries, data on use of these chemicals are limited or not available. To be most useful, use data would be provided for small geographic areas (e.g., county) and grouped by year. Because available use data are provided in a wide range of formats, creation of a centralized data aggregation system could aid researchers.

2. **Environmental monitoring—antimicrobials**: Studies examining persistence of selected antimicrobials and their metabolites are limited. Increased monitoring for these antimicrobials and their metabolites and degradation products in water, sediments, and other locations (e.g., air for triazoles) is needed to understand their environmental distribution. Monitoring of animal wildlife for tissue concentrations of these antimicrobials may be useful as well (Smalling & Fellers, 2013). Findings from such monitoring can be used in models to estimate distribution more widely. Triazoles in particular warrant further study, particularly given large increases in use over the past twenty years. Persistence of triazoles in the environment is often reported as days to weeks. However, triazoles may persist for months or longer in the environment, and environmental conditions heavily impact breakdown (Mosquera, 2010). In one of the few studies on the topic, conducted before their marked increase in use, triazoles were commonly detected in groundwater and surface water in the United States (Smalling & Reilly, 2013).

3. **Environmental monitoring—antimicrobial resistance**: Monitoring for antimicrobial resistance in environmental bacteria and fungi isolated in and around agricultural environments is also needed. These data would optimally be collected in the same settings as antimicrobial concentration data. Data on antimicrobial-resistance in bacteria and fungi that are not human pathogens may also be useful. For example, several *Aspergillus* species (e.g., *Aspergillus flavus*) are plant pathogens, and

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data are more widely available about triazole fungicide use surrounding plant pathogens than for the human pathogen *Aspergillus fumigatus*.

4. **Biomonitoring**: Little is known about the concentrations of the selected antimicrobials in human populations resulting from environmental exposures. Small studies have examined urinary concentrations of the fungicide tebuconazole and its metabolites in occupational settings (Fustinoni, 2014). Systematic analysis of human samples collected via existing biomonitoring systems could provide insight into the degree and possible sources of exposure. Such analysis would need to distinguish on a population level between medical antimicrobial use and other exposures. Experience with biomonitoring for tobacco use via cotinine levels suggests that distinguishing between direct use of a product and environmental exposure is feasible (Sexton, 2004).

5. **Public health surveillance for antimicrobial-resistant infections**: Although many factors influence antimicrobial resistance in human infections, public health surveillance for bacterial and fungal infections is essential for understanding the burden of resistance and for guiding studies examining links between environmental use of antimicrobials and resistant infections. Many examples of national and sentinel laboratory-based infectious disease surveillance exist. In the United States and Canada, no such broad-scale surveillance exists for *A. fumigatus* infections.

F.   What strategies that can be used to reduce or eliminate the need to use antimicrobials on crops?

By far, the best approach to limit the use of antimicrobials in plant production is through the use of the well-established measures of "Integrated Pest Management" (IPM), a systems approach designed to minimize economic losses to crops, as well as risks to people and the environment. The main components of IPM for plant diseases are 1) accurate diagnosis and monitoring, which can also include disease modeling and predictive systems to guide the timing of plant protection product applications; 2) use of disease resistant crop varieties, including resistant rootstocks in both fruit and vegetable systems; 3) exclusionary practices that prevent the introduction of pathogens into a crop such as using pathogen-free true seed and vegetative planting material, clean irrigation water and sanitation practices that prevent the movement of pathogens from plant-to-plant and field-to-field; 4) site selection and soil improvement to maximize plant health and minimize environmental factors that favor pathogens; 5) crop rotation and other cultural practices to prevent pathogen buildup; 6) use of biological and biorational products; and 7) judicious use of antibiotics and fungicides.

Consequently, growers use multiple methods, in addition to antibiotics, to control bacterial diseases of plants. Genetic resistance of host plants is the best method to control disease. This method is used for management of some bacterial diseases of vegetable and row crops. Unfortunately, for the destructive disease fire blight of pear and apple, breeding efforts have not yielded resistant fruiting cultivars (Norelli *et al.* 2003). The 'Red Delicious' apple is tolerant of fire blight, whereby floral infections kill fruiting spurs but the progression of the disease into stems was limited and the trees were not killed. Due to consumer demand, the 'Red Delicious' apple has been largely replaced by newer cultivars (e.g., 'Gala', 'Fuji', 'Honeycrisp', and others) that are susceptible to fire blight. All commercial pear cultivars are very susceptible to fire blight. Modern technologies, such as genomic sequencing, marker-assisted breeding and genome editing, could hasten the development of disease-resistant tree fruits and stone fruits (Norelli *et al.* 2003; Yang *et al.* 2012). While genetic modification of apple and pear for fire blight resistance may be possible, these trees could not be grown in orchards of certified organic growers. Furthermore, conventional growers may not invest in planting new orchards with genetically modified fruit trees without assurance that the fruit will be marketable and acceptable to consumers for decades into the future.

Cultural control methods are used routinely for management of bacterial diseases. For annual vegetable and row crops, cultural practices include crop rotation with plants that are not hosts for the bacterial disease of concern, using disease-free seeds and tubers, and soil solarization. For perennial crops, like fruit trees, crop rotations are not possible. For fruit trees, the location of the orchard can reduce the disease pressure of fire blight. The pear industry moved from the east coast of the US to the western states California, Washington and Oregon in the early 1900s. The warm, humid weather with frequent rain during the summer months in the eastern US were favorable for infections of pear flowers and subsequent infections of branches (shoot blight), resulting in complete loss of orchards (Thomson 2000). In the western states, the dry conditions during the summer months reduces the incidence of damaging secondary infections of stems by the fire blight pathogen.

Additional cultural control methods for bacterial diseases of fruit trees include sanitation (removal of diseased tissues and planting disease free plants), adjusting fertilizer applications to maintain plant health and to reduce vigor and production of succulent shoots, drip irrigation to reduce wetting of foliage and fruit, pruning to maintain good airflow through the canopy, and managing harmful insects that may spread bacteria or cause wounds that would serve as infection sites. While IPM practices are

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used by pear and apple growers for fire blight management, they are insufficient and additional tools are required for protection of tree fruits.

Non-antibiotic chemical control methods for fire blight management:

A mixture of hydrogen dioxide and peroxyacetic acid is available under the product name OxiDate 2.0 (BioSafe Systems, East Hartford, CT). This general biocide is registered for numerous crops to control fungal and bacterial diseases, including fire blight. The mixture of hydrogen dioxide and peroxyacetic acid kills bacteria on contact, but has little residual activity. Lime sulfur may be applied to apple trees during bloom to reduce the number of flowers and consequently the number of flowers that may be infected by the fire blight pathogen, but this material is not used on pear during bloom (Johnson and Temple, 2013). Copper compounds may be applied to dormant pear and apple trees and repeated during early bloom (Psallidas & Tsiantos 2000, Elkins *et al.* 2015). If copper is applied on pear and apple trees with young developing fruit, the fruit surfaces may be damaged due to phytotoxicity, resulting in spotted or misshapen fruit that have a reduced market value. New formulations of copper bactericides are less phytotoxic and can be used during late bloom for fire blight control with less potential for damaging fruit finish (Elkins *et al.* 2015).

Two additional chemicals, which are not bactericidal, are registered for fire blight management. Apogee (Prohexadione calcium, BASF Crop Protection, Research Triangle Park, NC) is a plant growth regulator that is registered for apple. Apogee reduces shoot growth and thus can reduce damaging secondary infections of succulent shoots by the fire blight pathogen; this damage is common in orchards exposed to humid summers and frequent rain such as the eastern US (Norelli *et al.* 2003). Actigard 50WG (Acibenzolar-S-methyl, Syngenta Crop Protection, Greensboro, NC) can reduce disease severity by inducing a natural process called systemic activated plant resistance. Actigard 50WG may be used therapeutically on infected trees by drenching the soil or painting the material on infected branches or trunks to reduce canker expansion (Johnson & Temple 2016, Johnson *et al.* 2016).

Biological control agents for fire blight:

Grower interest in biological control of fire blight increased with the widespread emergence of streptomycin-resistant populations of *E. amylovora* in apple and pear orchards in the western states (Loper *et al.* 1991; Jones & Schnabel 2000). The emergence of streptomycin-resistance destabilized antibiotic-based disease management programs and resulted in periodic epidemics, during which entire

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orchards were lost. Thousands of microbes isolated from orchards were screened for their ability to suppress growth of *E. amylovora* on flowers, thereby interrupting a key stage in the disease cycle (Johnson & Stockwell 1998; Lindow 1985, Pusey *et al.* 2009). Additional studies focused on the mechanisms of disease control of potential biological control agents and possible adverse effects to fruit quality from the biological control agents (Pusey *et al.* 2008; Stockwell *et al.* 2002; Wilson & Lindow 1993).

Currently, several biological control agents are registered for prevention of fire blight. Two *Bacillus*-based products are sold for management of fire blight. *Bacillus amyloliquefaciens* strain D747 (DoubleNickel LC, Certis, Columbia, MD) is registered for control of fungal and bacterial diseases on numerous crops, including pear and apple. *Bacillus subtilis* strain QST 713 (Serenade Max WDG or Serenade Opti, Bayer Crop Science LP, Research Triangle Park, NC) is sold as a spray-dried fermentation product containing the live organism and a mixture of lipopeptides produced in culture. The lipopeptides are essential for efficacy; growth of the bacterium on plant surfaces is not required for disease control. Serenade Opti is applied just prior to predicted infection periods, similar to the timing of antibiotic applications, but numerous applications are recommended for disease control.

Several other biological agents manage fire blight by a mechanism called pre-emptive exclusion (Wilson & Lindow 1993). In pre-emptive exclusion, nutrients for pathogen growth are depleted by the biocontrol agent and the pathogen is excluded from sites for colonization and infection. The biocontrol agents must be applied during early to mid-bloom to give the biocontrol agent time to grow to large population sizes prior to floral colonization by the pathogen. Three biological control agents that operate in part by pre-emptive exclusion are BlightBan A506 (*Pseudomonas fluorescens* strain A506, NuFarm Americas, Burr Ridge, IL), Bloomtime FD (*Pantoea agglomerans* strain E325, NuFarm Americas, Burr Ridge IL), and Blossom Protect (*Aureobasidium pullulans* strains DSM 14940 and DSM 14941 suspended in an acidic buffer, Westbridge Agricultural Products, Vista, CA). In addition to pre-emptive exclusion, the bacterium in Bloomtime FD produces an uncharacterized secondary metabolite on flowers that is toxic to *E. amylovora* (Pusey *et al.*, 2011). An advantage of biological control agents is that, unlike antibiotics, they grow and spread among flowers; that is, the biocontrol bacteria spread from colonized flowers to newly opened flowers that may not have been protected by earlier chemical sprays (Johnson *et al.* 2000; Lindow & Suslow 2003). Well-timed applications of the bacterial biological control agents during bloom can significantly reduce the incidence of fire blight under low to moderate disease pressure (Johnson *et al.* 1993; Lindow 1985; Stockwell *et al.* 2010).

Challenges to implementation of biological control

Use of biological control agents requires grower education and changes in how they approach fire blight management. Instead of using traditional decision aids to determine the need for disease control measures and the timing of intervention, growers need to commit during early bloom to a biologically-based disease control program to permit establishment and growth of the biological control agents prior to arrival of the pathogen to flowers. Furthermore, growers need to apply the biological control agents during conditions that support growth of the organism (Johnson *et al*. 2000). A decision-aid for use of biological control agents was developed to guide the timing of applications to maximize the potential for successful establishment and growth prior to migration of the pathogen to flowers (Johnson *et al*. 2004).

The biological control agents generally work best in the western states where bloom progresses over one to three weeks and conditions are moderately warm to support growth of the organism. In other regions of the US, bloom occurs rapidly and environmental conditions during early bloom are often too cold to support rapid growth of the biological control agents, which may decrease control efficacy (Sundin *et al*. 2009).

Variability in consistency in performance of biological control agents across environments is another impediment to widespread adoption of this technology (Johnson & Temple 2013; Sundin *et al*. 2009). In some years or locations, the biological control agents perform well, but in other years they may fail to control disease(Stockwell *et al*. 2010). Additionally, while excellent disease control is reported with Blossom Protect (Johnson & Temple, 2013), the yeasts may cause russet or mark fruit finish on certain cultivars of pear and apple during cool, wet environmental conditions (Johnson & Temple, 2013). Russet mars the fruit finish and decreases the fresh market value of the fruit. Consequently, some growers hesitate to use Blossom Protect, especially in orchards in regions with cool, wet spring weather. Additionally, the yeasts in Blossom Protect are sensitive to copper and many of the fungicides used to control scab, powdery mildew and other fungal diseases in orchards. The incompatibility of Blossom Protect with many fungicides adds an extra level of complexity for management of fruit orchards during bloom to fruit development (Johnson & Temple, 2013; Sundin *et al*. 2009).

In summary, antibiotics have been used for decades for control of two serious plant diseases, fire blight of pear and apple and bacterial spot of peach and nectarine without documented deleterious effects to the environment or animal and human health (McManus, 2014). IPM practices have reduced the number of antibiotic applications needed to manage fire blight and bacterial spot. Antibiotics are

applied primarily when warm weather coincides with full bloom in orchards with a recent history of disease in the orchard or nearby. If these conditions are not met, antibiotics are not applied. In the US, organic-certified growers are at the forefront of testing if antibiotic-free commercial fruit production is feasible because antibiotic registrations for organic pear and apple production were withdrawn in October 2014. Given that fire blight epidemics generally occur every 5 to 10 years within a fruit producing region, the capacity to control diseases, like fire blight, without antibiotics likely will be subjected to real-world tests within the coming decade.

III.   LITERATURE REVIEW

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ABBREVIATIONS

AMR: antimicrobial resistance

CDC: U.S. Centers for Disease Control and Prevention

ECDC: European Centre for Disease Prevention and Control

EPA: U.S. Environmental Protection Agency

MRL:

HGT: horizontal gene transfer

MDR: multi-drug resistance

MIC: minimum inhibitory concentration

Glossary

Antibiotics: Traditionally refers to natural organic compounds produced by microorganisms that act in low concentrations against other microbial species, mostly bacteria. Sometimes, the terms "antibiotics" is used to refer to synthetic (chemotherapeutic) and semi-synthetic compounds (chemically modified antibiotics) with similar effects.

Antimicrobial agents: A general term for the drugs (antibiotics), chemicals, or other substances that either kill or inhibit the growth of microbes. The concept of **antimicrobial agents** applies to antibiotics, disinfectants, preservatives, sanitizing agents, and biocidal products in general. All antibiotics are **antimicrobial agents**, but not all **antimicrobial agents** are antibiotics.

Antimicrobial resistance: A property of microorganisms that confers the capacity to inactivate or exclude antimicrobials, or a mechanism that blocks the inhibitory or killing effects of antimicrobials.

Biocide/Biocidal products: Active substances and preparations containing one or more substances intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means.

Biocide resistance: When non-antibiotic antimicrobial agents (i.e., biocides) are considered, the word "resistance" is used in a similar way when a strain is not killed or inhibited by a concentration attained in practice (the in-use concentration) and in a situation where: 1) a strain is not killed or inhibited by a concentration to which the majority of strains of an organism are susceptible, or 2) bacterial cells are not killed or inhibited by a concentration acting upon the majority of cells in that culture (SCENHR 2009).

Co-resistance: Resistance occurring when the genes specifying different resistant phenotypes are genetically linked, for example by being located together on a mobile genetic element (e.g., a plasmid, transposon, or integron).

Co-selection: Occurs when non-medically important antibiotic selects for resistance to a medically important antibiotic because resistance to both antibiotics are genetically linked.

Cross-resistance: Resistance occurring when the same or similar mechanism(s) of resistance applies to different antimicrobials.

Heavy metal: Naturally occurring elements that have a high atomic weight and a density at least 5 times greater than that of water.

Heavy metal resistance: Bacteria are considered to be resistant to heavy metals when: 1) a strain is not killed or inhibited by a concentration to which the majority of strains of a organism are susceptible, or 2) when bacterial cells that are not killed or inhibited by a concentration acting upon the majority of cells in that culture.

Horizontal gene transfer (HGT): Transfer of genetic material between bacterial cells due to other processes than cell division. E.g. transduction, transformation, and transduction.

Intrinsic resistance: A natural property of an organism resulting in the absence of or a decreased susceptibility to a particular antimicrobial agent.

Isolate (bacteria/fungi): A bacterial/fungal isolate is a single isolation in pure culture from a specimen.

Minimum Inhibitory Concentration (MIC): The lowest concentration of a given agent that inhibits growth of a microorganism under standard laboratory conditions. MIC data can provide information about the activity of antimicrobials.

Selection: A process by which some bacterial/fungal species or strains of bacteria/fungi in a population are selected for due to having a specific growth or survival advantage over other microorganisms.

Strain: A subset of a bacterial/fungal species differing from other bacteria/fungi of the same species by some minor, but identifiable, difference.

Susceptibility: Describes the extent to which an antimicrobial agent affects a target microorganism.

Transferable resistance: Antimicrobial resistance that can be transferred between microorganisms and their mobile-encoded resistant genes can be next transferred to other microorganisms.

DRAFT